Effects of simulated anaemia on the blood chemistry of Atlantic cod, Atlantic halibut, and Atlantic salmon

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Foreword

This project was completed to fulfill the requirements of the Master of Science (MSc) in Aquaculture as outlined by the Faculty of Biosciences and Aquaculture, Bodø University College. The thesis is a supervised individual project worth 60 credit points. This project focuses on the areas of aquatic health and welfare.

The objective of this thesis was to develop a multi-species protocol for the induction of a stable and reproducible experimental anaemia, as well as to characterize any changes in haematology and blood chemistry of anaemic fish.

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Abstract

Anaemia is a common pathology associated with many diseases and infections, though its physiological effects are poorly understood. This project aimed to develop a methodology for inducing a stable experimental anaemia in Atlantic cod, halibut, and salmon, and thereby investigate the physiological impact of anaemia.

Atlantic cod, halibut, and salmon were rendered progressively anaemic by means of intraperitoneal injections of 3 μg·g-1 phenylhydrazine in a dimethyl sulfoxide vehicle, dissolved in Cortland's saline. One injection was sufficient to induce anaemia in Atlantic cod, while two injections, one week apart, provided an acceptable reduction in haematocrit and haemoglobin for both Atlantic halibut and salmon.

There was a significant reduction in plasma lactate in the phenylhydrazine-injected group of Atlantic cod, and plasma glucose was significantly reduced in anaemic and control fish for both Atlantic halibut and salmon. Additionally, there was a significantly lower concentration of plasma sodium and chloride in the Cortland's saline-only control group of Atlantic halibut. Peripheral blood smear analysis showed no significant differences in leukocyte proportions in any of the species investigated, but there was a marked increase in immature erythrocytes in the anaemic groups of all species.

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1.0 Introduction

1.1 Anaemia

Anaemia is a common pathology in aquaculture that can be associated with many different causes. The severity of its presentation can be dictated by the type of anaemia, such as a visible skin discoloration associated with haemolytic anaemia (Beutler, 2005). Also determining the extent to which the anaemia becomes a hindrance to the fish are the lifestyle and habits of the fish- more active fish would likely have different/more severe effects from the same extent of anaemia when compared with their less active, benthic counterparts.

The mode in which anaemia presents clinically can differ between species and the underlying cause. However, the general physiological effect of anaemia is likely to be the dysfunction of oxygen transport (Prchal, 2005). The lowered ability of the blood to transport oxygen around the body has the result that the supply of oxygen to the tissues will be limited. This is because the partial pressure of the oxygen in the capillaries is insufficient to offload the oxygen to the cells. As a result, there is a decrease in oxygen consumption at the cellular level, with the cells depending more heavily on less efficient glycolysis for ATP generation (Prchal, 2005). Another potential effect of anaemia is a decreased oxygen affinity at the surface of the erythrocyte. Small decreases in pH induce the Bohr effect, and cause the erythrocytes to release their oxygen (Prchal, 2005). Under chronic stress, there can be a change in the concentration of β-adrenergic receptors on the RBC surface (Perry, Reid, and Salama, 1996). Whether increased or decreased, however, appears to depend on the predominant type of circulating "stress" hormone, with high cortisol levels generally increasing the receptor concentration, while catecholamines serve to reduce the number of receptors (Perry et al., 1996).

Hypoxia-inducible transcription factor 1α (HIF-1α) is an important part of the defences against hypoxia, as it serves to regulate the transcription of erythropoietin (Prchal, 2005). This means that under anaemic conditions there can be an increase in red blood cell production. Also, the animal may compensate for lower oxygen efficiency by increasing cardiac output (Prchal, 2005). This may come in the form of increased stroke volume, frequency, or even a remodelling of the heart itself (Simonott and Farrell, 2007).

There are many potential causes of anaemia in the aquatic environment. Malnutrition and disease are two main causes of anaemia in fish. Malnutrition with respect to specific vitamins, for example, thiamine, pyridoxine, folic acid, inositol, biotin, choline, niacin, and vitamins E and K, has been documented as having the potential to cause anaemic effects (Pillay and Kutty, 2005). With regard to disease, anaemia is a common pathological sign of many different diseases. Examples include infections with copepods, parasites, lice, or cestodes, as well as infectious salmon anaemia (ISA), viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN), spring viraemia of carp (SVC), channel catfish viral disease (CCVD), coldwater vibriosis, rainbow trout fry anaemia, to name a few (Woo, Bruno, and Lim, 2003).

1.2 Anaemia models

Several models have been developed to investigate experimental anaemia in various species. The most common models have generally been induced physically by removing red blood cells from circulation (controlled bleeding), or injecting the test animal with a chemical compound. These methods induce different types of anaemia- haemorrhagic anaemia in the case of controlled bleeding, and haemolytic anaemia in the case of chemical injection.

1.2.1 Haemorrhagic anaemia

Haemorrhagic anaemia results directly from the loss of blood at a rate greater than the body's ability to produce new cells (Roberts and Rodger, 2001). Controlled bleeding of a test subject can be employed to induce an anaemic state. This method has been widely used in many experiments involving a variety of vertebrates, ranging from rats (Bozzini, Boyer, Friedman, Lezón, Norese, and Alippi, 1998) to teleost fishes (Wood, McDonald and McMahon, 1982). Wood, McMahon and McDonald (1979, 1982) employed a method of bleeding fish through the caudal vein and centrifuging the blood to separate it into its fractions. Once this was completed, they eventually replaced the plasma and "buffy coat" portions, supplemented with a sufficient amount of saline solution to match the initial; blood volume. They repeated this process several times, with small amounts of blood so as to gradually induce the anaemic state (Wood et al., 1979). Replacing the volume of blood removed with an equivalent volume of saline is necessary to avoid a decrease in blood pressure.

1.2.2 Haemolytic anaemia

Haemolytic anaemia is the result of haemolysis, or a shortening of the lifespan of the red blood cells. There are many known chemical causes of haemolytic anaemia, including arsenic, lead, copper, and chlorates (Beutler, 2005). Generally induced experimentally by administering a chemical or drug with haemolytic effects, the anaemia should begin to diminish once the source compound and its metabolites are removed from the system. The different agents have various modes of affecting the erythrocyte; arsenic interacts with sulfhydral groups, lead inhibits erythrocyte enzymes, including those involved in porphyrin metabolism, copper can inhibit enzymes and catalyzes the oxidation of intracellular reduced

glutathione (GSH). In some cases, such as with chlorates, the affected erythrocytes can produce Heinz bodies within the cell (Beutler, 2005).

1.3 Phenylhydrazine-induced anaemia

Phenylhydrazine has been frequently used for its haemolytic properties since its discovery by Fischer in 1875 (Berger, 2007). It has been noted to induce an anaemic condition in various species including human, dog, frog, rabbit (Cary, Dobson, and Brooke, 2000), rat (Dornfest, Bush, Lapin, Adu, Fulop, and Naughton, 1990), and various species of fish including pinfish, *Lagodon rhomboids* (Cameron and Wohlschlag, 1969), chinook salmon, *Oncorhynchus tshawytscha* (Smith, McLain, and Zaugg, 1971), and rainbow trout, *Oncorhynchus mykiss* Walbaum (Simonott and Farrell, 2007, Jones, 1971).

Phenylhydrazine (PHZ) works as a redox reactive drug, reacting with the haemoglobin of erythrocytes, and creating destructive by-products such as benzene, hydrogen peroxide, superoxide anion, and the phenyl radical (Shetlar and Hill, 1985). The speed at which the reaction occurs is critically dependent upon the amount of oxygen available, as one molecule of unbound O_2 is consumed per PHZ reaction (Shetlar and Hill, 1985). Contrary to other cases of haemolytic anaemia in which there is a change in the cell membrane structure (an externalization of phosphatidylserine, a signal for phagocytosis), haemolysis by PHZ is the result of increased activity of reactive oxygen species (Berger, 2007). Overall, the administration of phenylhydrazine results in a decrease in total haemoglobin and a reduction of both red blood cell quantity and packed cell volume. An increase in the amount of haemoglobin per erythrocyte has been seen experimentally, as well as an elevated number of reticulocytes (Berger, 2007). A study in rats indicated that phenylhydrazine can also act to

promote a proliferation of lymphoid cells, effectively stimulating the immune system (Dornfest et al. 1990).

1.4 Aims

This thesis aimed to develop a method for inducing a stable and reproducible anaemic state that is applicable for multiple species. Further, it investigated the changes in basic blood chemistry parameters that occur in anaemic fish. The progressive changes in haematocrit and haemoglobin concentrations, plasma lactate and glucose content, electrolyte content, and white blood cell profiles were tracked in groups of anaemic and control fish, and inter- as well as intra-treatment differences were investigated. It was expected that there would be a marked decrease in haematocrit and haemoglobin contents in phenylhydrazine-injected fish, as well as an increase in plasma lactate, as has been seen in many anaemia studies (Cameron and Wohlschlag, 1969; Olsen, Falk and Reite, 1992; Wood, McDonald, and McMahon, 1982). Plasma glucose was expected to increase, as a result of the secondary stress response (Iwama, Afonso, and Vijayan, 2006). A disruption in electrolytes would likely be apparent due to the stress of acid imbalance (Lee, Gerking, and Jezierska, 1983). Changes in the proportions of white blood cells were also expected to be seen in the phenylhydrazine-injected anaemic fish, as other researchers have noted incidences of leukocytosis (in rat: Dornfest, Lapin, Naughton, Adu, Korn, and Gordon, 1986).

2.0 Materials and Methods

2.1 Fish Treatment

All fish used in the experiments were acclimated in 2 x 2 x 0.4 m tanks for a minimum of two weeks prior to the start of experimentation. The tanks were supplied with degassed seawater from 250 m depth at a temperature of 7- 9 ºC and oxygen saturation > 90%, under a 12L: 12D photoperiod. The Atlantic cod (*Gadus morhua*) used were obtained from Codfarmers AS, Gildeskål, Norway. The Atlantic halibut (*Hippoglossus hippiglossus*) were two-year old fish obtained from Mørkved Bukta Research Station, reared on-site. The Atlantic salmon (*Salmo salar*) were originally obtained from the Mainstream AS facility at Hopen, Norway, and were provided by the Mørkved Bukta Research Station.

At the start of each experiment, randomly selected fish were individually removed from the holding tanks with a dipnet and placed into a bath of light anaesthetic (60 $mg \cdot l^{-1}$ MS- 222, Sigma-Aldrich, Oslo Norway). For each fish, the wet weight was measured to a specificity of 0.1 g, the total length was recorded to the nearest 1 mm, and an individually numbered and colour-coded t-bar tag (Floy tag company, Seattle WA, USA) was inserted into the dorsal fin to allow for identification of individual treatments. A blood sample (of 250-500 µl volume, depending on the experiment) was taken from the caudal vein with a 23- gauge needle, treated with 5000U ml^{-1} ammonium heparin (Sigma-Aldrich, Oslo Norway). Immediately following this, the fish were injected intraperitoneally via 23-gauge needle with a volume of phenylhydrazine (PHZ)/ dimethylsulfoxide (DMSO)/ Cortland's saline solution, DMSO/ Cortland's saline solution, or Cortland's saline solution, which was determined based on the individual mass. Following injection, the fish were recovered in fresh seawater, until

equilibrium was regained and they exhibited regular opercular movement, before they were returned to the holding tank. All fish in a given experiment were held in a single tank.

From the collected blood samples, smaller volumes of 200 and 20 µl were removed for haematocrit and haemoglobin analyses, respectively. A blood smear was also prepared for each fish by spreading a small drop of blood on a microscope slide. The remainder of the blood was centrifuged at 8 000 rpm for approximately two minutes and the plasma was aspirated, kept on ice, and frozen at -20ºC for later analysis.

2.1.1 Atlantic cod

2.1.1.1 Experiment one and two

The purpose of these first experiments was to determine the dose of phenylhydrazine necessary to induce a significant and sustainable anaemic state. In experiment one, a dose of 10 $\mu g \cdot g^{-1}$ PHZ, which had previously been used in rainbow trout (Simonott and Farrell, 2007), was injected into ten randomly selected fish (average length 33.1 ± 3.7 cm, weight 384.3 ± 119.0 g). A blood sample of 200 µl was taken on day zero (injection day), day two, and day six. The second experiment was designed to determine an optimal dose of phenylhydrazine for Atlantic cod. Six fish (30.7 ± 3.2 cm, 325.7 ± 72.8 g) were injected with a saline control, six fish (33.4 ± 3.2 cm, 410 ± 196.1 g) with 2.5 $\mu g \cdot g^{-1}$, eight fish (31.6 ± 3.1 cm, 309 ± 101.7 g) with 5 $\mu g \cdot g^{-1}$, and six fish (32.3 ±3.5 cm, 320.5 ± 126.5 g) with 8 $\mu g \cdot g^{-1}$. The fish were sampled at day zero, and again after two, seven, and 14 days. Blood smears were not prepared for these samples.

2.1.1.2 Experiments three and four

From experiments one and two, a dosage of 3 $\mu g \cdot g^{-1}$ phenylhydrazine provided an optimal stable and reproducible anaemic state. The next step was to investigate the longer-term effects of the drug. Consequently, in the third experiment, 15 fish (average length 32.0 ± 3.3 cm, weight 311.3 ± 70.9 g) were injected with 3 $\mu g \cdot g^{-1}$ PHZ/DMSO/Cortland's saline, 15 fish (32.2 ± 2.4 cm, 327.9 ± 71.5 g) with DMSO/Cortland's saline, and six fish (32.4 ± 2.9 cm, 314.5 ± 110.4 g) with Cortland's saline. Blood sampling took place at day zero (injection), and subsequently at days three, seven, and 14. The fourth experiment was identical in setup to the third, but blood samples were taken less frequently, at days zero, 14, and 21.

2.1.2 Atlantic halibut

To determine the dose of PHZ necessary for the halibut, blood samples were taken from two fish to inspect the normal percentage of haematocrit. A conservative estimate of 3 $\mu g \cdot g^{-1}$ PHZ/ DMSO/ Cortland's saline was injected into 12 randomly selected fish (average length 45.5 ± 2.7 cm, weight 1138.0 ± 267.1 g). Twelve fish (average length 45.9 ±2.6 cm, weight 1182.6 ± 190.9 g) were injected with DMSO/Cortland's saline, five fish (average length 44.3 ± 6.1 cm, weight 1031.8 ± 471.1 g) received an injection of Cortland's saline only, and an initial blood sample of 500 µl was taken. On day seven post-injection, the haematocrit of three PHZ-group fish were tested to gauge the severity of the anaemia. The average packed cell volume was still 15.3%, which was above the desired target value of 10% or less. The fish were subsequently injected with a further 3 $\mu g \cdot g^{-1}$ of PHZ/DMSO/Cortland's saline solution. The fish were blood sampled again on days 14, 21, and 28.

2.1.3 Atlantic salmon

Twenty-five (average length 32.4 ± 2.5 cm, weight 366.9 ± 87.2 g) randomly selected salmon were injected intraperitoneally with 3 $\mu g \cdot g^{-1}$ PHZ/DMSO/Cortland's saline, 20 fish (33.5 ± 2.6 cm, 404.4 ± 124.5 g) with DMSO/Cortland's saline, and seven fish (32.2 ± 1.7 cm, 356.2 ± 60.6 g) with a Cortland's saline control. An initial blood sample of 500 µl was taken. Blood samples were taken at day zero (from initial experiment start date), day 8, 15, 22, and day 26. On the 8th day, haematocrit in the PHZ-injected group was not sufficiently low (mean 29.57% ± 1.30 SE), so all fish were injected a second time with their respective solutions.

2.2 Phenylhydrazine preparation

To prepare the PHZ/ DMSO/ Cortland's saline solution, 0.03 g each of both phenylhydrazine and dimethyl sulfoxide were weighed in a beaker, and enough Cortland's saline was added to reach a final weight of 5 g. The DMSO/ Cortland's saline solution was prepared in a similar manner, without the addition of PHZ. The solutions were kept in 2 ml plastic tubes, and new solutions were prepared fresh before each experiment.

2.3 Haematocrit analysis

Haematocrit was measured using a micro haematocrit centrifuge (Heraeus Pico 17, Thermo Scientific, Copenhagen Denmark). Duplicate 100 µl heparinised micro haematocrit tubes (BRAND, Germany) per fish were filled with whole blood and centrifuged for five minutes at 13,500 rpm. The packed cell volume was determined visually using a haematocrit reader card. The volume was measured to the nearest half percent, and an average of the two tubes was taken.

2.4 Haemoglobin analysis

For haemoglobin analysis, 20 µl of whole blood was fixed in 1 ml of Drabkin's Solution (Sigma-Aldrich, Oslo Norway). A series of haemoglobin standards was prepared using bovine haemoglobin (Sigma-Aldrich, Oslo Norway), and a standard curve constructed. Fifty microlitres of the blood-Drabkin's mixture was diluted with 200 µl of fresh Drabkin's solution to achieve a final dilution factor of 1:5, as required by the procedure provided. The absorbance of the resulting solutions was measured at 540 nm using a microplate reader (Tecan Sunrise, Tecan Nordic, Denmark). The concentration of haemoglobin in each sample was calculated from the standard curve.

2.5 Lactate and Glucose

Plasma lactate was measured using an AccuTrend hand-held lactate meter with BM-Lactate test strips (Roche, Mannheim Germany). Hand-held meters have been used in a number of recent studies (Scott, Wood, Sloman, Iftikar, De Boeck, Almeida-Val, Val, 2008; Brown, Watson, Bourhill, and Wall, 2008), with good results compared to other analytical methods. When available, measurements were made using whole blood as the sample material. However, many of the measurements were performed on plasma samples. In such cases, a standard curve was prepared for each new pack of test strips, to account for inter-pack differences. The high- and low-point measurements of the supplied standards were measured with the device on "Plasma Mode", and standard curves were constructed. The device had a lower detection limit of 0.7 $mmol \cdot l^{-1}$ and an upper limit of 26 $mmol \cdot l^{-1}$ in plasma mode. A value of zero was used in calculations for samples that had lactate contents below the measurable limit.

Glucose was measured using an Ascensia Contour hand-held glucose meter with Ascensia Microfill glucose test strips (Bayer, Oslo, Norway). The device was self-calibrating and able to handle both whole blood and plasma as the sample material and had a lower detection limit of 0.6 $mmol \cdot l^{-1}$, and an upper limit of 33.3 $mmol \cdot l^{-1}$. Plasma was the main material used, except in the final round of salmon blood sampling when whole blood was used as the medium.

2.6 Electrolyte analysis

Plasma sodium, chloride, and potassium were measured using a VetStat Electrolyte and Blood Gas Analyzer (IDEXX, Kruuse Norge AS, Drøbak, Norway). Preliminary tests indicated that a dilution was required prior to analysis. The samples were diluted 2:1 with deionized water. Each sample was individually aspirated into the sample chamber and the electrolyte concentration values were recorded.

2.7 Blood smears

A peripheral blood smear was prepared for the initial (pre-injection) and terminal blood samples of the Atlantic cod from experiment four, as well as for the Atlantic halibut and salmon. The slides were allowed to completely air dry before they were fixed in methanol, stained with Wright's stain, and cover slipped. The slides were inspected visually with the aid of a microscope under 40-60X magnification and the number and type of white blood cells (WBCs) counted. A minimum of 100 WBCs per slide was counted from non-contiguous fields. Representative samples (Figures 1-3) of the cells counted in each cell category were photographed. The relative proportion of immature red blood cells was also noted.

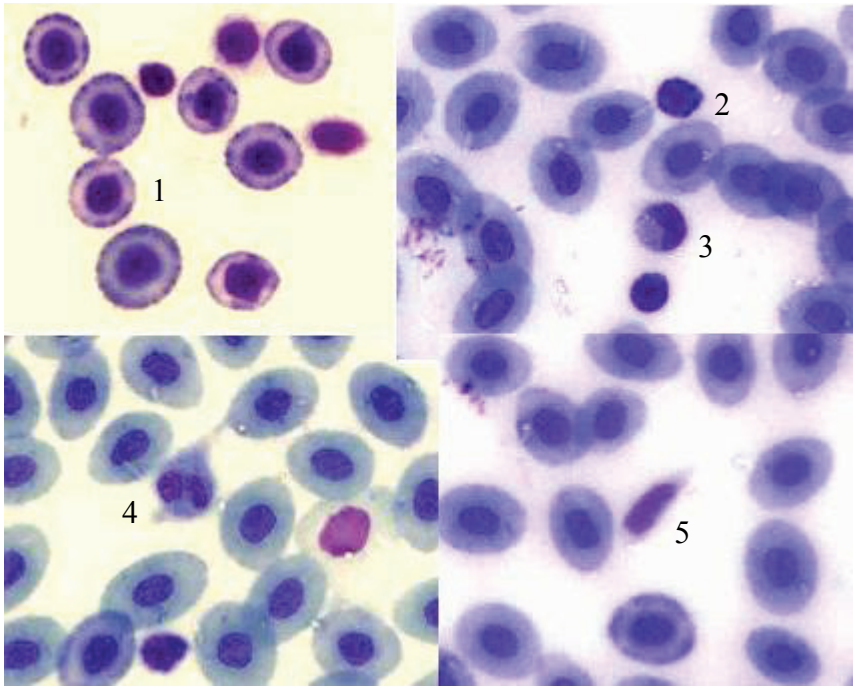

Figure 1- Atlantic cod blood cells, 60x magnification. Representative samples of immature erythrocytes (1), lymphocytes (2), monocytes (3), neutrophils (4), and thrombocytes (5).

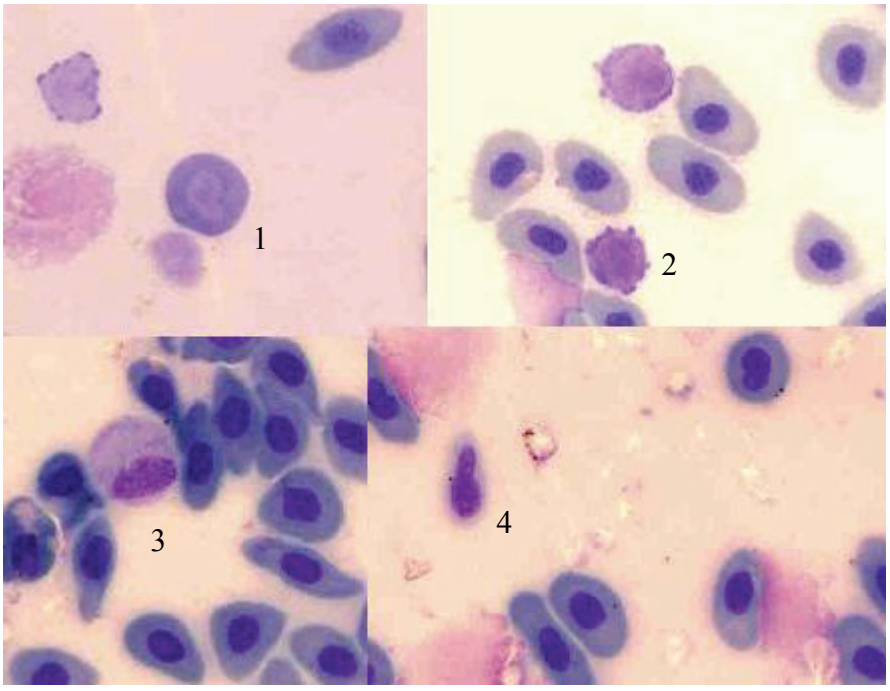

Figure 2- Atlantic halibut blood cells, 60x magnification. Representative samples of immature erythrocytes (1), lymphocytes (2), monocytes (3), and thrombocytes (4). No neutrophils were counted in this species.

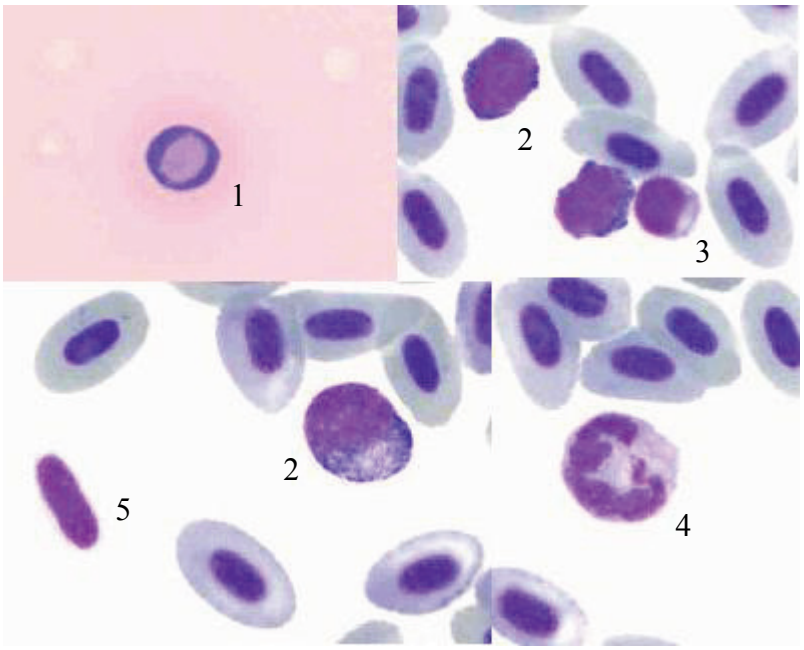

Figure 3- Atlantic salmon blood cells, 60x magnification. Representative samples of immature erythrocytes (1), lymphocytes (2), monocytes (3), neutrophils (4), and thrombocytes (5).

2.8 Statistical analysis

Data analysis was completed using SigmaPlot 10 (Systat Software, Chicago, USA). All data was analyzed using one-way ANOVAs, with a Holm-Sidak method for multiple pairwise comparisons. P-values of less than 0.05 were considered significant. When data was not normally distributed or non-homogenous, a Kruskall-Wallis one-way ANOVA on ranks was used, followed by Dunn's Method for pairwise comparisons. For two-group comparisons, Student's t-test was used, or a Mann-Whitney U test when the equal variance assumption was not met. Z-tests were used to compare the relative proportions of white blood cells in the blood smear section.

3.0 Results

3.1 Haematocrit, haemoglobin, and mortality

3.1.1 Atlantic cod experiments one and two

3.1.1.1 Haematocrit

Initial, pre-treatment haematocrit values ranged from 22.9 to 28.3%. At the end of the experiment, the values ranged from 14.9-21.4%. There was a significant reduction in haematocrit (Figure 1) after two days in both the 8 and 10 $\mu g \cdot g^{-1}$ PHZ groups (t_7= 5.746, P= < 0.001; t_8= 9.521, P= < 0.001). There was no significant reduction in the 5 $\mu g \cdot g^{-1}$ ($F_{3,13}$= 2.045, P= 0.157), 2.5 $\mu g \cdot g^{-1}$ ($F_{3,15}$= 2.819, P= 0.075) or 0 $\mu g \cdot g^{-1}$ ($F_{3,18}$= 1.624, P= 0.219) groups during the course of the experiment. There was a significant ($F_{2,10}$ 6.044 P= 0.025) difference between the final haematocrit values (after 14 days) between the different treatments (Figure 4). The 8 and 10 $\mu g \cdot g^{-1}$ PHZ groups were not included in the comparison, as there were no survivors (Figure 5).

3.1.1.2 Mortality

There were heavy losses during this experiment, particularly at the higher doses (Figure 5). There was a 100% mortality rate for the two highest doses, 8 and 10 $\mu g \cdot g^{-1}$. There were mortalities in each group, with the 0, 2.5, and 5 $\mu g \cdot g^{-1}$ groups sustaining 22, 33, and 75% losses, respectively (Figure 5). The deaths in the 0 $\mu g \cdot g^{-1}$ control group occurred immediately after injection, and there were no further losses.

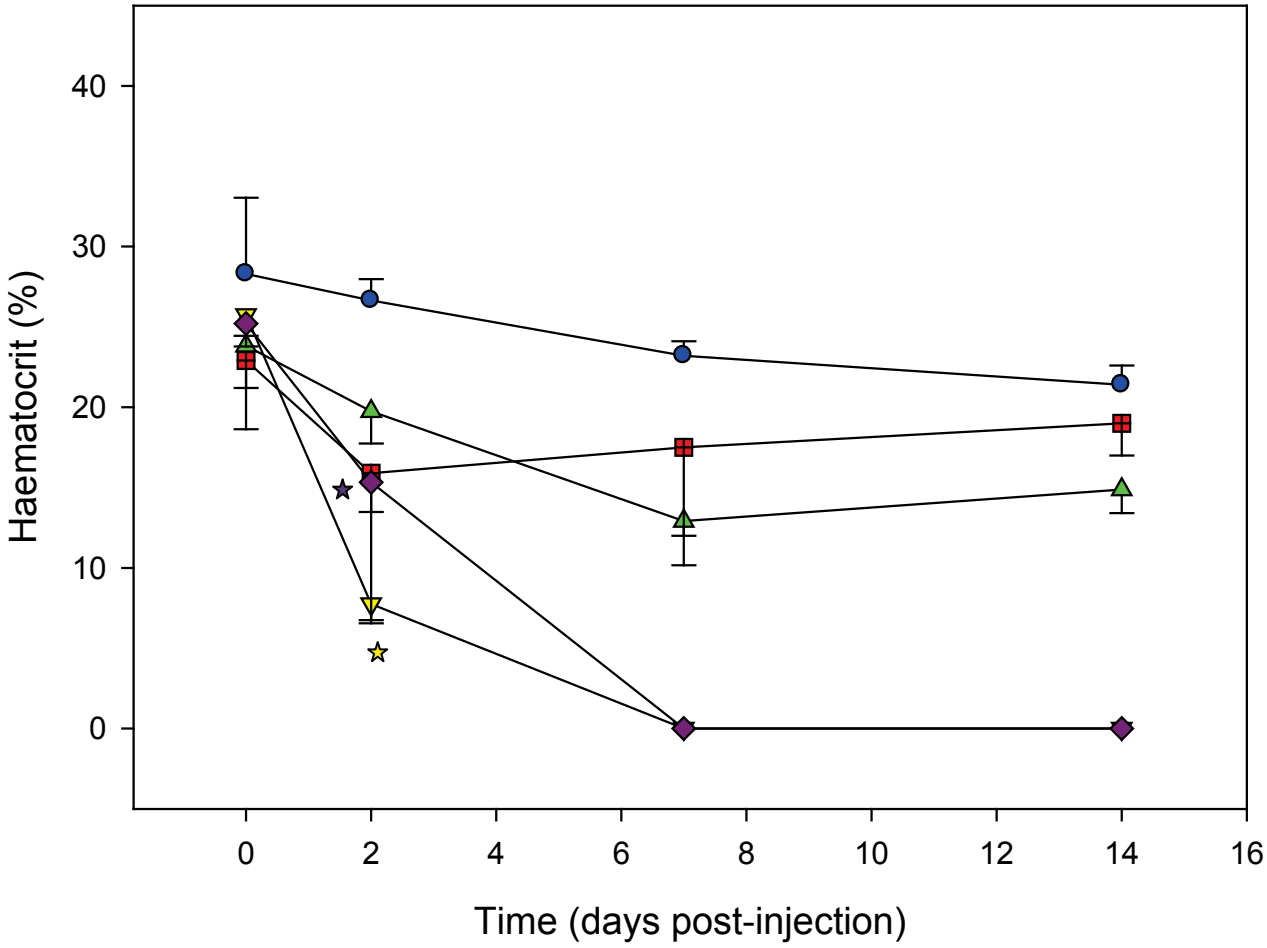

Figure 4- Haematocrit progression, Atlantic cod (*Gadus morhua*) experiments 1 and 2. The 10 µg·g^{-1} group is represented by diamonds; 8 µg·g^{-1} by inverted triangles; 5 µg·g^{-1} by squares; 2.5 µg·g^{-1} by triangles, and 0 µg·g^{-1} by circles. Significant deviations from the initial haematocrit concentrations are represented by a star. The only differences seen were in the second samples from the 8 and 10 µg·g^{-1} groups. (After this point, all individuals in these groups were deceased)

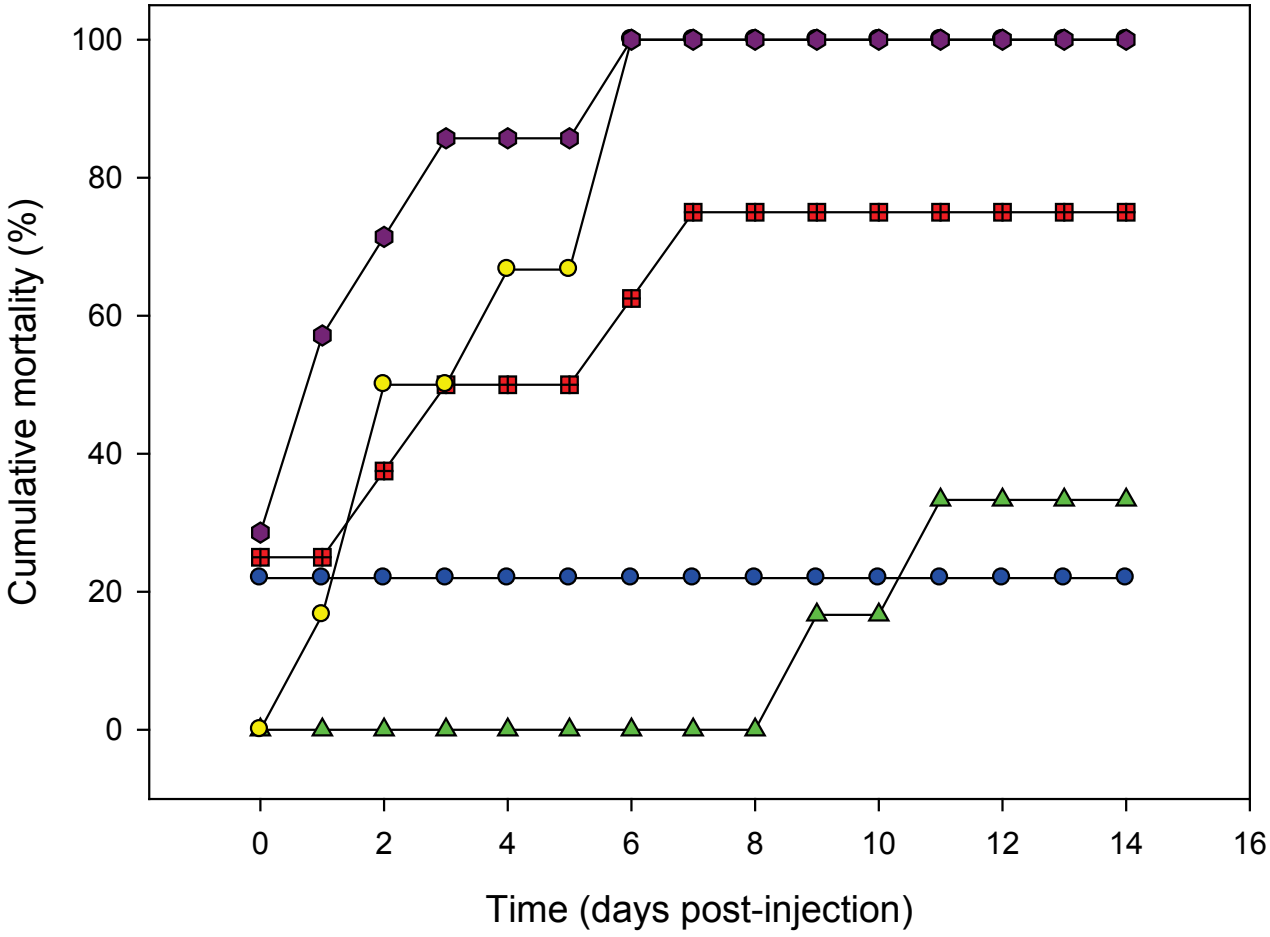

Figure 5- Cumulative mortality, Atlantic cod (*Gadus morhua*) experiments 1 and 2. The 10 µg·g^{-1} group is represented by filled hexagons; 8 µg·g^{-1} by light circles; 5 µg·g^{-1} by filled squares; 2.5 µg·g^{-1} by triangles, and 0 µg·g^{-1} by dark circles.

3.1.2 Atlantic cod experiment three

3.1.2.1 Haematocrit

The mean pre-treatment haematocrit concentrations ranged from 27.1 ± 1.68 - 29.1 ± 0.67% (Figure 6). At the end of the experiment, the values ranged from 8 ± 0.00- 24.4 ± 0.95%. The phenylhydrazine-injected group showed variation in haematocrit concentrations throughout the experiment, with the samples from days 3, 7, and 14 having significantly ($F_{2,29}$= 60.365, P= < 0.001) lower haematocrit values than the initial sample (Figure 6). The DMSO group also had significant differences ($F_{2,38}$= 6.478, P= < 0.001) in haematocrit concentration in the samples from days 7 and 14, when compared to the initial value. No differences, however, were seen in the saline-only group. There was also a significant difference ($F_{2,19}$ 23.134 P= < 0.001) between terminal haematocrit percentages between the three treatments (Figure 6). There were slight (16 and 11%, respectively) reductions in haematocrit in the DMSO/Cortland's saline and Cortland's saline groups, while The PHZ group had a reduction of 71%, from an initial haematocrit of 27.7 ± 1.04 % to 8 ± 0.00%.

3.1.2.2 Haemoglobin

Mean pre-injection haemoglobin concentrations ranged from 6.52 ± 0.44 - 8.65 ± 0.55 $g \cdot dl^{-1}$, and the terminal means ranged from 2.51 ± 0.72- 5.90 ± 0.37 $g \cdot dl^{-1}$ (Figure 7). The haemoglobin content followed a similar pattern as the haematocrit concentrations (Figure 7). There was a significant difference (H_3= 33.094, P= < 0.001) in haemoglobin in the phenylhydrazine-injected group, with the samples from days 3, 7, and 14 having a lower content of haemoglobin than the initial. There was also a difference in the DMSO/Cortland's saline group, in that the samples from days 7 and 14 had significantly ($F_{3,53}$= 6.598,

P= < 0.001) lowered haemoglobin (Figure 7). No differences were seen in the saline-only group ($F_{3,18}$= 1.773, P= 0.131). When the treatments were compared, the phenylhydrazine-injected group had significantly lower haemoglobin compared to the other treatments ($F_{2,18}$= 10.955, P= < 0.001).

Mean Corpuscular Haemoglobin Concentration

The mean corpuscular haemoglobin concentration (MCHC) decreased significantly (H_3= 8.263, P= 0.041) between the second and third samples in the PHZ-injected group, from a mean of 33.22 $g \cdot dl^{-1}$ ± 2.80 SE to 22.14 ± 5.50 $g \cdot dl^{-1}$ (Figure 8). It then showed a small, but non-significant increase at the final sampling point, to a mean of 22.86 $g \cdot dl^{-1}$ ± 5.09. In the DMSO group, the final MCHC was substantially lower than those of the first and second samples (H_3= 12.220, P= 0.007). This treatment group started with an MCHC of 29.68 $g \cdot dl^{-1}$ ± 1.75, increased slightly to 30.83 $g \cdot dl^{-1}$ ± 1.91 by day 3, then progressed to a low of 24.01 $g \cdot dl^{-1}$ ± 0.79 after the 14-day experiment (Figure 8). The saline-only group started with an MCHC of 24.68 $g \cdot dl^{-1}$ ± 2.67, increased to 28.48 $g \cdot dl^{-1}$ ± 2.12 by day 3, decreased to a low of 21.68 $g \cdot dl^{-1}$ ± 2.57 at day 7, and finished with an MCHC of 22.26 $g \cdot dl^{-1}$ ± 0.78. These changes, however, were non-significant (H_3= 7.091, P= 0.069).

3.1.2.3 Mortality

There was one mortality in the DMSO/ Cortland's saline group immediately following injection, and no further mortalities (Figure 9). No fish were lost in the saline control group, while there was a cumulative loss of 80% in the PHZ treatment group.

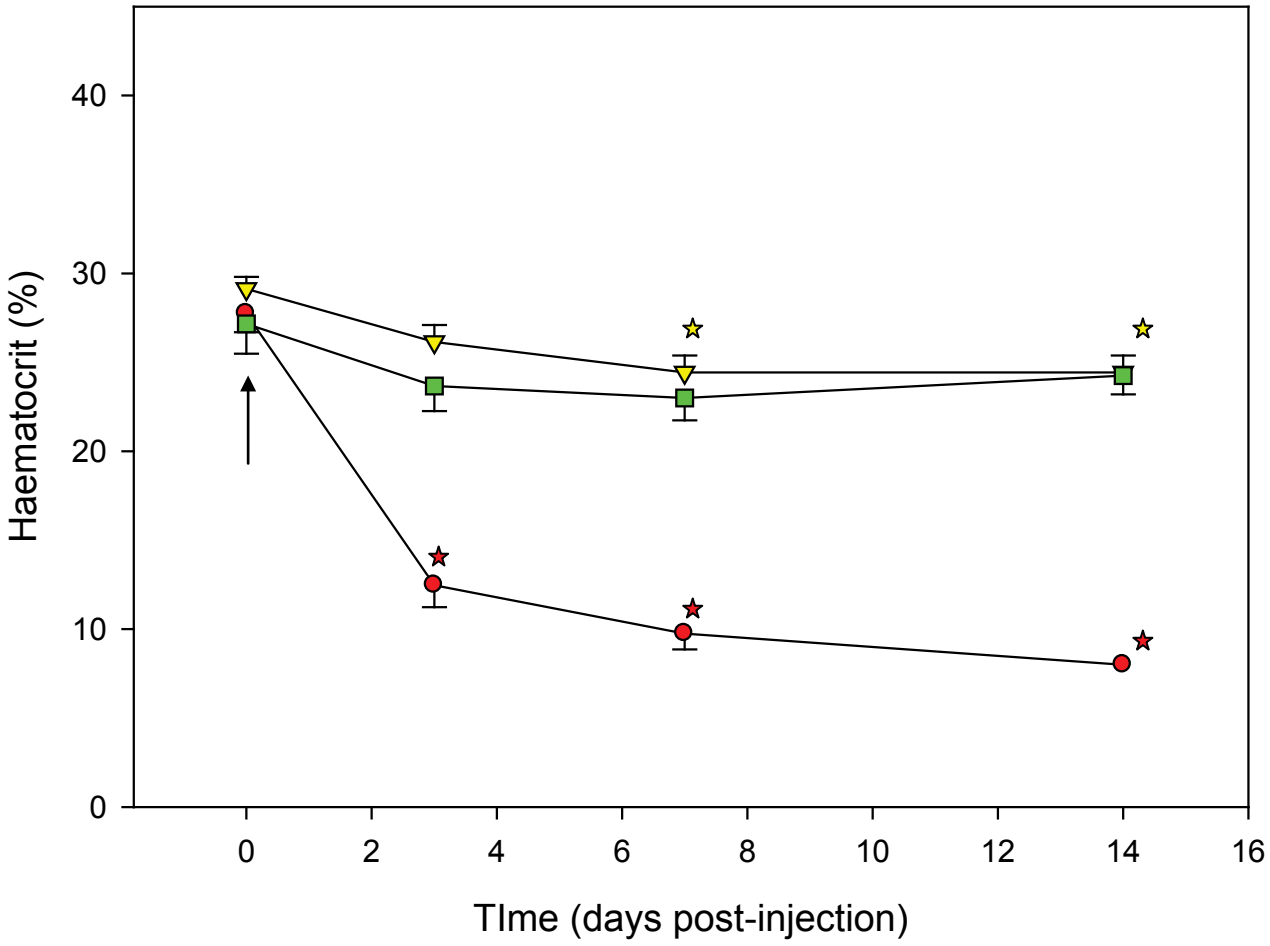

Figure 6- Haematocrit progression, Atlantic cod (*Gadus morhua*) experiment 3. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. Arrows indicate injection dates, and stars represent haematocrit concentrations that are significantly different from the initial. In the phenylhydrazine group, all haematocrit concentrations were significantly different from the initial, and in the dimethyl sulfoxide group, the samples from days 7 and 14 were significantly different from the initial. There were no significant differences seen in the Cortland's saline-only group.

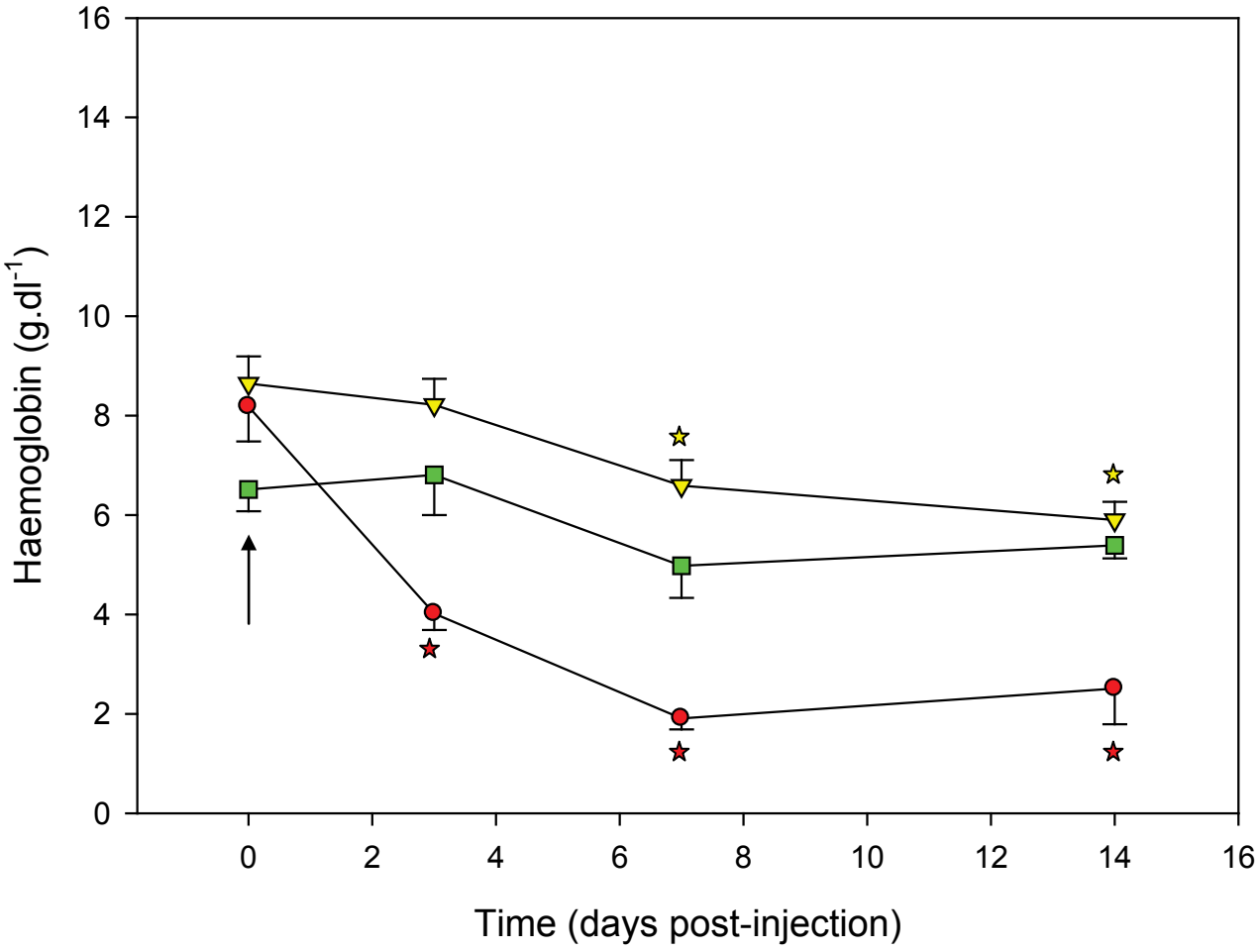

Figure 7- Haemoglobin progression, Atlantic cod (*Gadus morhua*) experiment 3. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. Arrows indicate injection dates and stars indicate values that are significantly different from initial values. All subsequent samples in the phenylhydrazine group were different than the initial, and the samples from days 7 and 14 were significantly different in the dimethyl sulfoxide group. There were no differences seen in the Cortland's saline-only group.

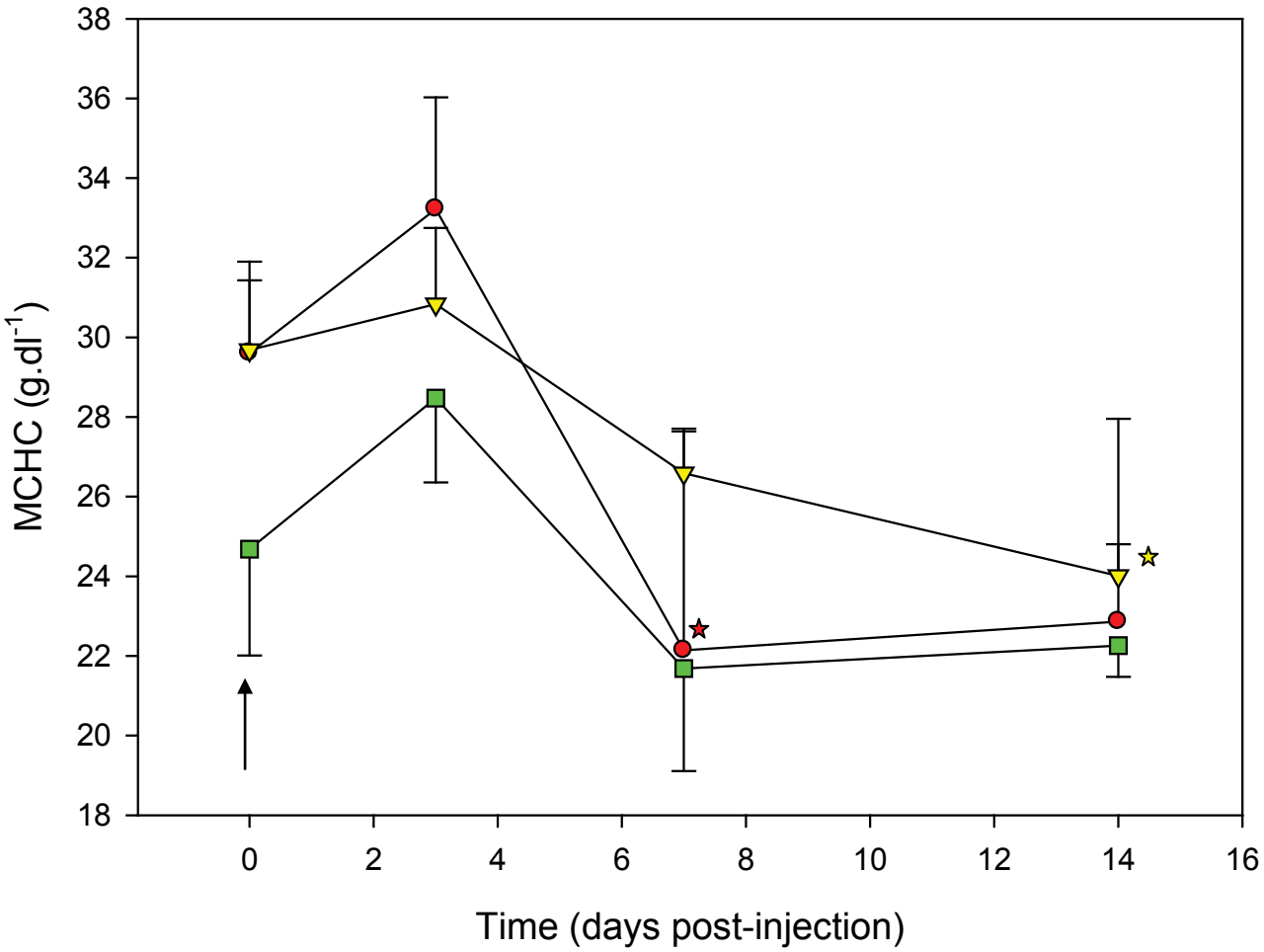

Figure 8- Mean corpuscular haemoglobin concentration changes throughout Atlantic cod (*Gadus morhua*) experiment 3. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. The arrow indicates the date of injection, and stars indicate values that are significantly different. There was a significant decrease in the phenylhydrazine-injected group between days 3 and 7. In the dimethyl sulfoxide group, the sample from day 14 is significantly different than the samples from days zero and 3. No changes were seen in the Cortland's saline-only group.

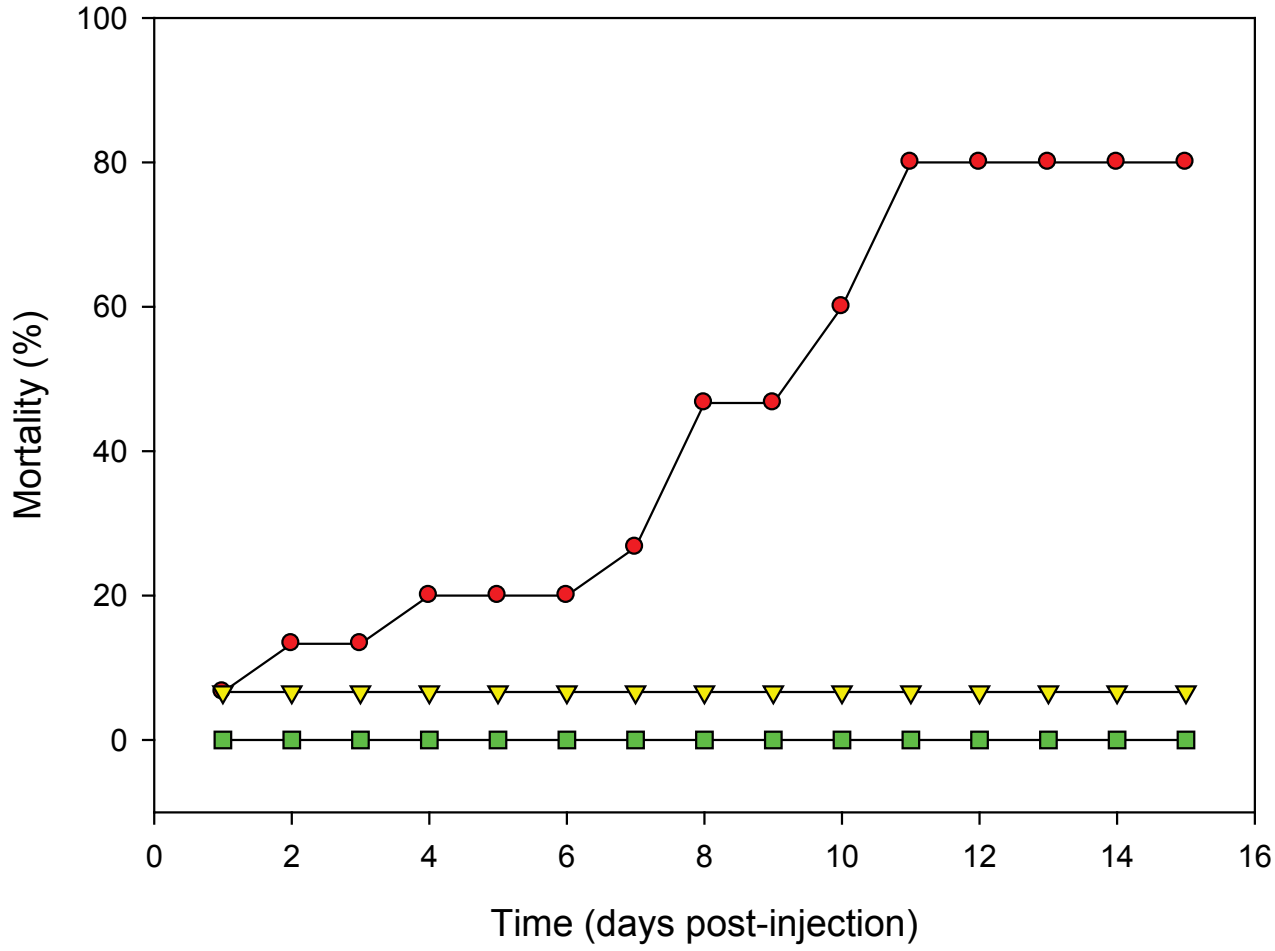

Figure 9- Cumulative mortality, Atlantic cod (*Gadus morhua*) experiment 3. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares.

3.1.3 Atlantic cod experiment four

3.1.3.1 Haematocrit

Initial mean haematocrit values ranged from 28.1 ± 3.68 SE - 29.2 ± 2.62% (Figure 10). The final values ranged from 17.9 ± 4.01- 25.0 ± 4.14%. The phenylhydrazine-injected group had significant variations in haematocrit ($F_{2,29}$= 63.805, P= < 0.001), with the samples from days 14 and 21 having lower concentrations than the initial (Figure 10). In the DMSO group, the sample from day 21 had a lower haematocrit concentration than the initial ($F_{2,38}$= 5.345, P= 0.009). No differences ($F_{2,12}$= 2.242, P= 0.149) were seen in the saline-only group, though. There was a significant difference ($F_{2,22}$= 7.611 P= 0.003) between terminal haematocrit values for the different treatments (Figure 10).

3.1.3.2 Haemoglobin

Before treatments, the mean haemoglobin was between 6.31 ± 0.36 SE - 7.06 ± 0.37 $g \cdot dl^{-1}$, which reduced to between 3.10 ± 0.45 - 6.21 $g \cdot dl^{-1}$ ± 0.68 by the end of the experiment (Figure 11). There were significant differences ($F_{2,27}$= 29.798, P= < 0.001) in haemoglobin values for the PHZ group throughout the experiment (Figure 11). However, there were no differences in the DMSO (P= 0.323) or saline-only (P= 0.116) groups. When compared to each other, the effect of treatment was apparent, as the phenylhydrazine-injected group had a significantly ($F_{2,22}$= 5.457, P= 0.012) lower final haemoglobin content- 3.10 $g \cdot dl^{-1}$, when compared to the DMSO group (5.30 $g \cdot dl^{-1}$) and the Cortland's saline-only group (4.49 $g \cdot dl^{-1}$).

Mean Corpuscular Haemoglobin Content

There were no significant changes in MCHC in any of the treatment groups throughout the duration of this experiment (Figure 12). The PHZ group started with an MCHC of

20.79 $g \cdot dl^{-1}$ ± 1.65 SE, which progressed to a low of 17.97 ± 1.45 after 14 days, and increased to 18.28 ± 5.54 at day 21 (H_2= 5.351, P= 0.069). The DMSO group showed less variation throughout the sampling dates, from the initial 23.22 $g \cdot dl^{-1}$ ± 0.63, increasing to 24.95 ± 0.53, then down to 24.85 ± 2.59 after 21 days (H_2= 3.117, P= 0.210). There was no significant difference in MCHC in the Cortland's saline-only group ($F_{2,12}$= 0.511, P= 0.613), with an initial concentration of 24.38 ± 1.85, and a concentration of 19.06 $g \cdot dl^{-1}$ ± 5.06 at day 21 (Figure 12).

3.1.3.3 Mortality

There was an initial, post-injection mortality of 20% in the PHZ treatment group, 6.7% in the DMSO group, and 16.7% in the saline group (Figure 13). After 21 days, there was 53% mortality in the PHZ treatment group, 20% in the DMSO group, and 33% in the Cortland's saline control group.

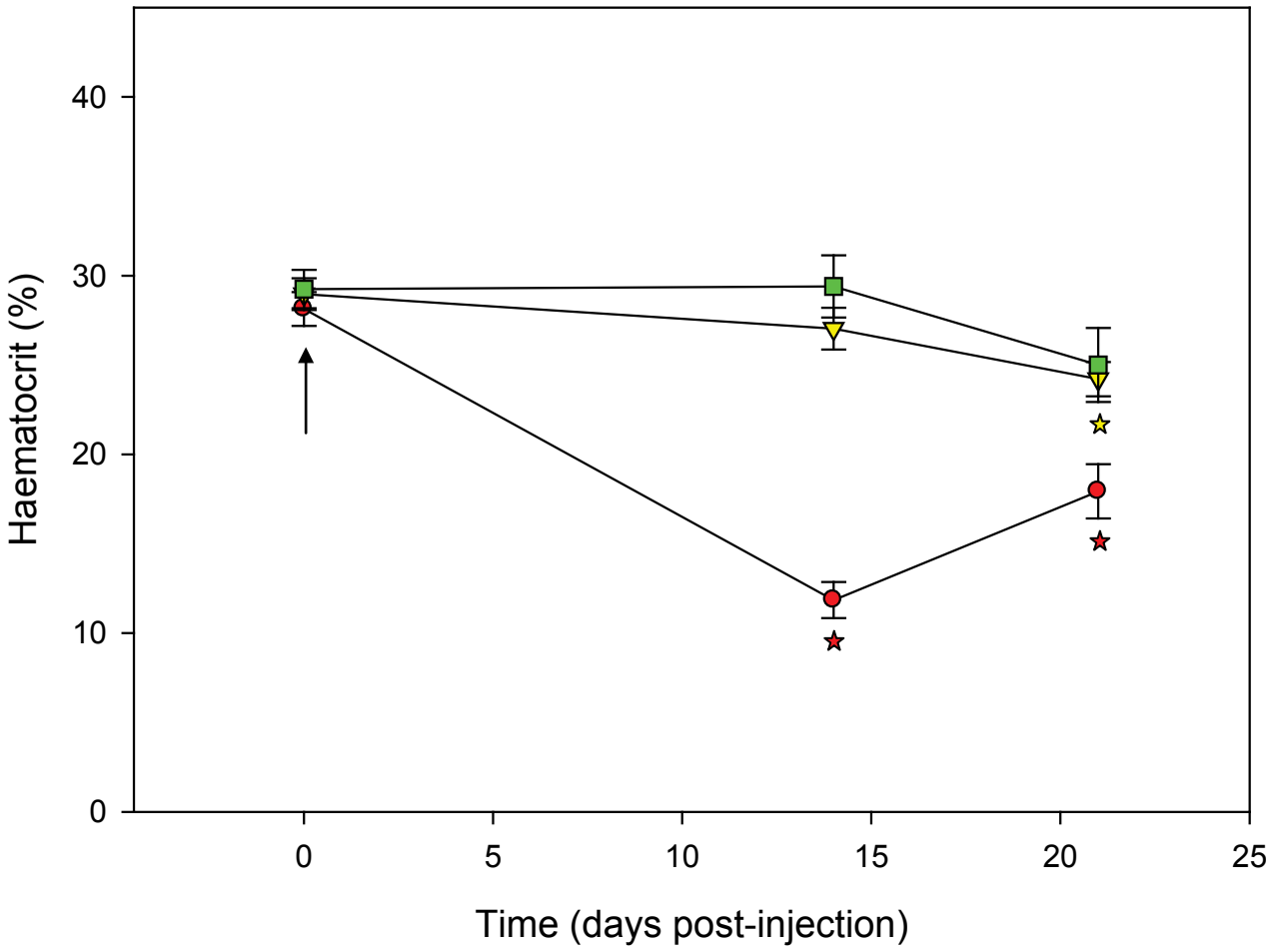

Figure 10- Haematocrit progression, Atlantic cod (*Gadus morhua*) experiment 4. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. Arrows represent injection dates, and stars indicate haematocrit concentrations that are significantly different from the initial. For the phenylhydrazine group, the samples from days 14 and 21 were significantly different from day zero, and the haematocrit concentration on day 21 in the dimethyl sulfoxide group was significantly lower than the initial. There were no differences seen in the Cortland's saline-only group.

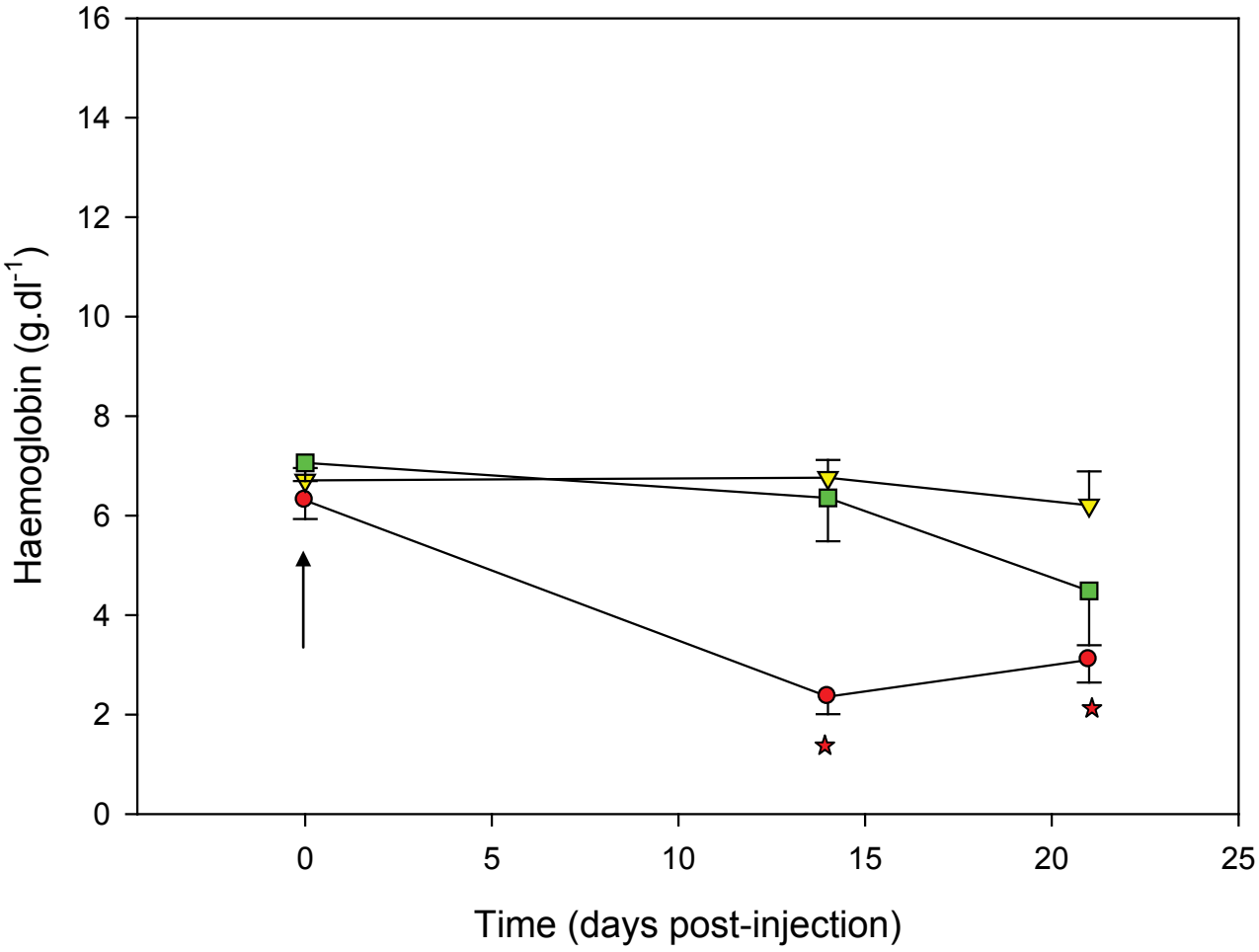

Figure 11- Haemoglobin progression, Atlantic cod (*Gadus morhua*) experiment 4. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. Arrows represent injection dates and stars represent values that are significantly different from initial values. The samples from days 14 and 21 from the phenylhydrazine group had lower haemoglobin content than the initial.

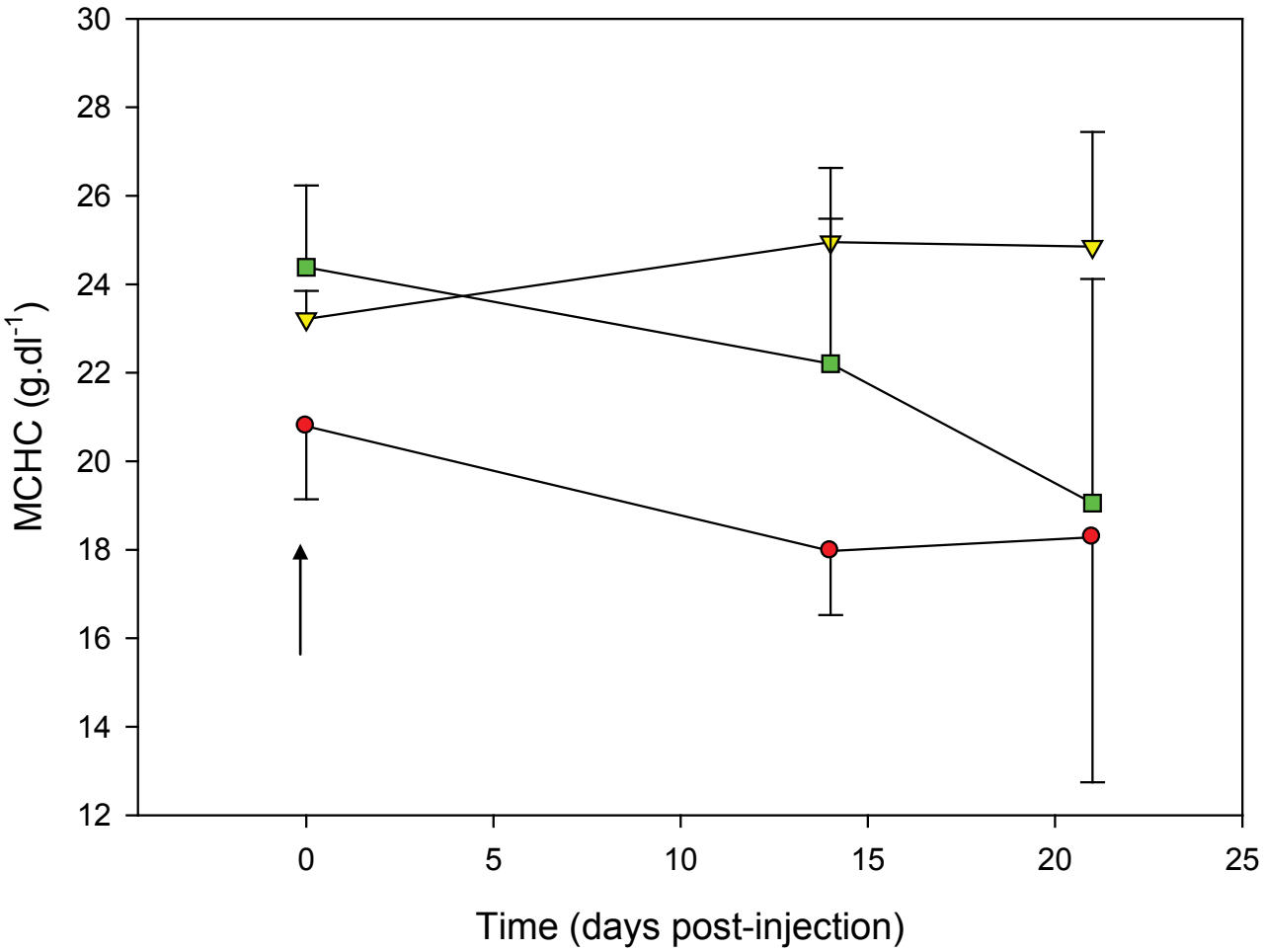

Figure 12- Mean corpuscular haemoglobin concentration changes throughout Atlantic cod (*Gadus morhua*) experiment 4. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. The arrow indicates the injection date. There were no significant differences in MCHC throughout the experiment.

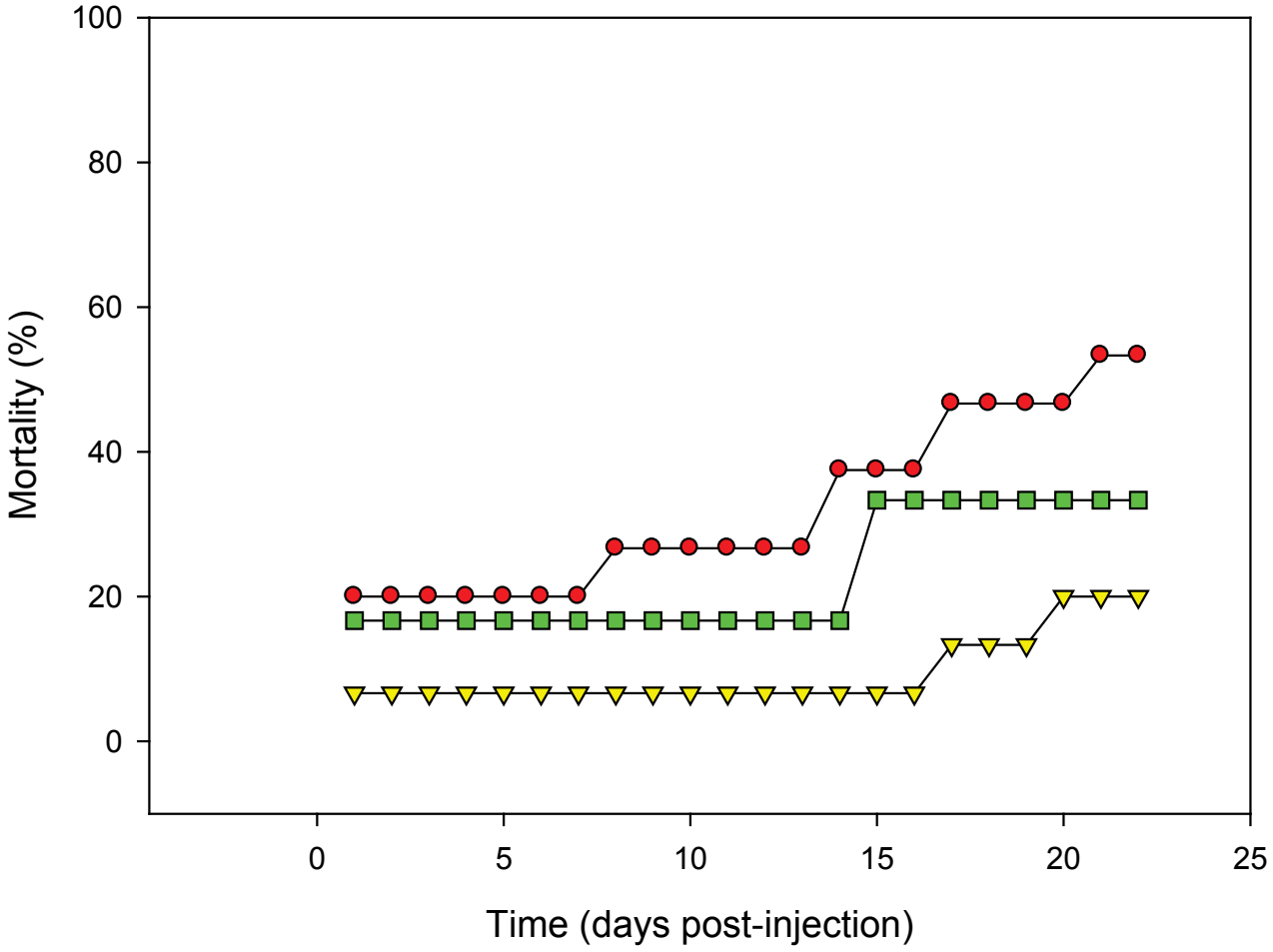

Figure 13- Cumulative mortality, Atlantic cod (*Gadus morhua*) experiment 4. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares.

3.1.4 Atlantic halibut

3.1.4.1 Haematocrit

There was significant variation between haematocrit concentrations in all of the treatment groups throughout the experiment (Figure 14). The mean pre-injection haematocrit ranged from 30.50 ± 1.77 SE- 31.88 ± 0.85%. The end-of-experiment mean haematocrit percentages ranged from 7.56 ± 0.60- 25.5 ± 0.74%. In the PHZ group, the samples from days 14, 21, and 28 had significantly lower haematocrit values than the initial (H_4= 39.520, P= < 0.001). There were significant differences ($F_{4,55}$= 6.117, P= < 0.001) in all sampling instances for the DMSO group, when compared to the initial concentration. There was a difference between the initial and final (day 28) haematocrit values only for the Cortland's saline-only group ($F_{4,20}$= 4.495, P= 0.009). In addition, there was a significant difference ($F_{2,25}$= 138.077 P= < 0.001) between the final haematocrit values for the three treatments (Figure 14).

3.1.4.2 Haemoglobin

The haemoglobin content shows a similar progression as was seen for haematocrit (Figure 15), with initial mean values of 12.16 ± 0.36 SE- 12.48 ± 0.44 $g \cdot dl^{-1}$, and final contents of 2.55 ± 0.28- 10.4 ± 0.35 $g \cdot dl^{-1}$. All post-injection sampling instances show significantly lower haemoglobin content in the phenylhydrazine group ($F_{4,46}$= 71.429, P= < 0.001). The samples from days 21 and 28 in the DMSO group had lower haemoglobin concentrations compared to the initial ($F_{4,53}$= 4.346, P= 0.004), and the final samples from the saline-only group also had lower amounts of haemoglobin ($F_{4,20}$= 3.553, P= 0.024). When the different treatments were compared, the phenylhydrazine-injected group had a significantly lower ($F_{2,21}$= 151.944, P= < 0.001) haemoglobin content than either the DMSO or saline-only groups (Figure 15).

Mean Corpuscular Haemoglobin Concentration

There was a considerate difference (H_4= 22.309, P= < 0.001) in MCHC values in the phenylhydrazine-injected group throughout the course of the 28-day experiment (Figure 16). The group started with an MCHC of 39.39 $g \cdot dl^{-1}$ ± 0.59 SE, which increased greatly to 45.05 ±1.28 by day 7. The MCHC remained elevated at the next two sample instances, and starkly decreased to 33.82 $g \cdot dl^{-1}$ ± 2.55 by day 28 (Figure 16). In the DMSO treatment group, there was a substantial (H_4= 12.742, P= 0.013) increase in MCHC between the first and second samples, from 39.12 ± 0.71 to 44.21 $g \cdot dl^{-1}$ ± 1.68. This was the only significant change, and the final concentration was 41.63 $g \cdot dl^{-1}$ ± 1.41. There were no significant changes (H_4= 2.496, P= 0.645) in the MCHC of the saline-only group, which had a start concentration of 40.47 ± 0.65 and ended at 42.40 $g \cdot dl^{-1}$ ± 4.18 at day 28 (Figure 16).

3.1.4.3 Mortality

There were no mortalities in either the DMSO or the Cortland's saline-only groups (Figure 17). Three mortalities occurred on day 13 in the phenylhydrazine group, for a total loss of 25%. There were no further mortalities in the experiment.

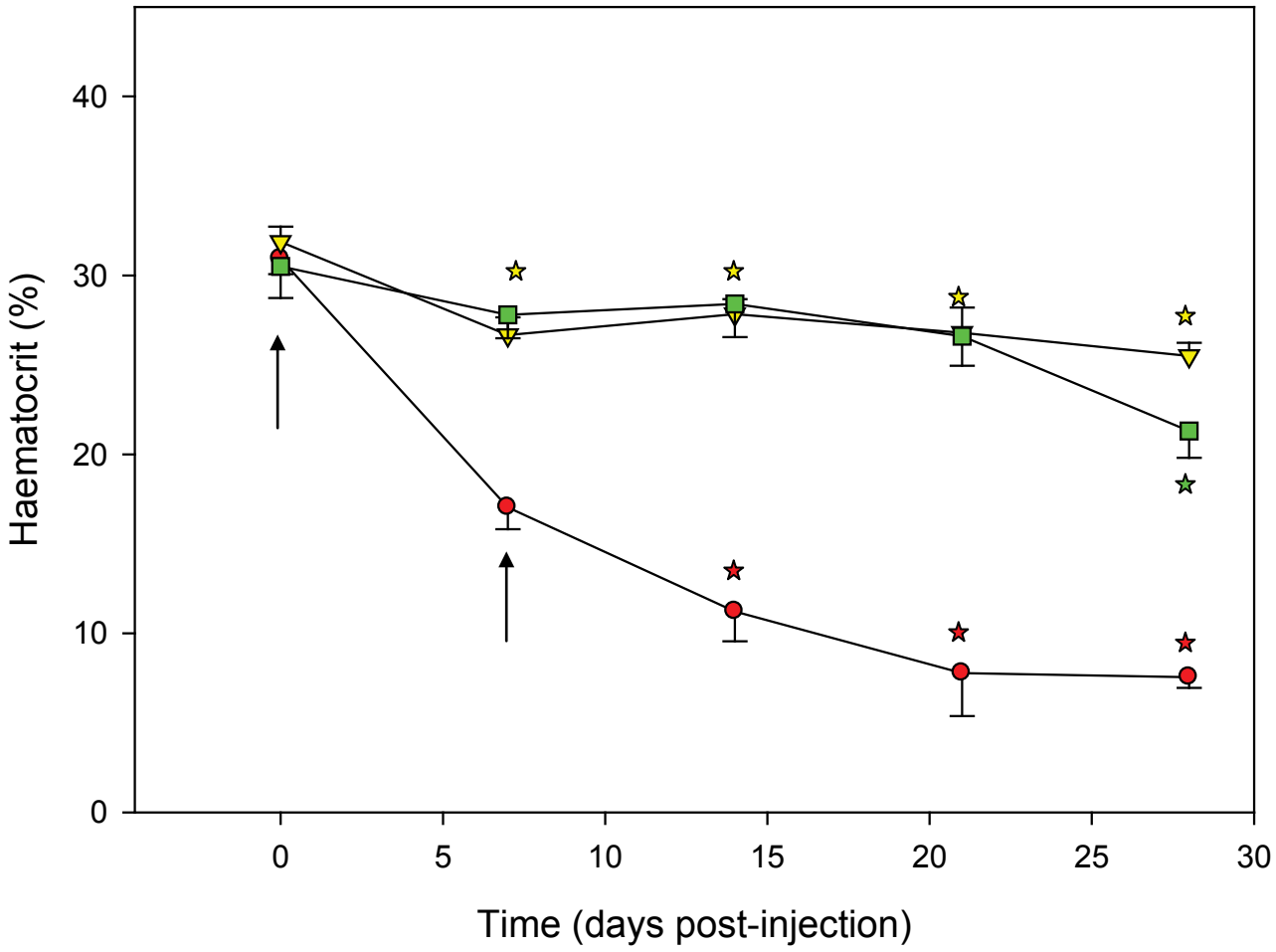

Figure 14- Haematocrit progression, Atlantic halibut (*Hippoglossus hippoglossus*) experiment. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. Arrows indicate injection dates. Stars indicate values that are significantly different from initial concentrations. The phenylhydrazine group had significant differences after 14, 21, and 28 days; all samples were significantly different from the initial in the dimethyl sulfoxide group, while only the final sample in the Cortland's saline-only group had a significantly different haematocrit percentage.

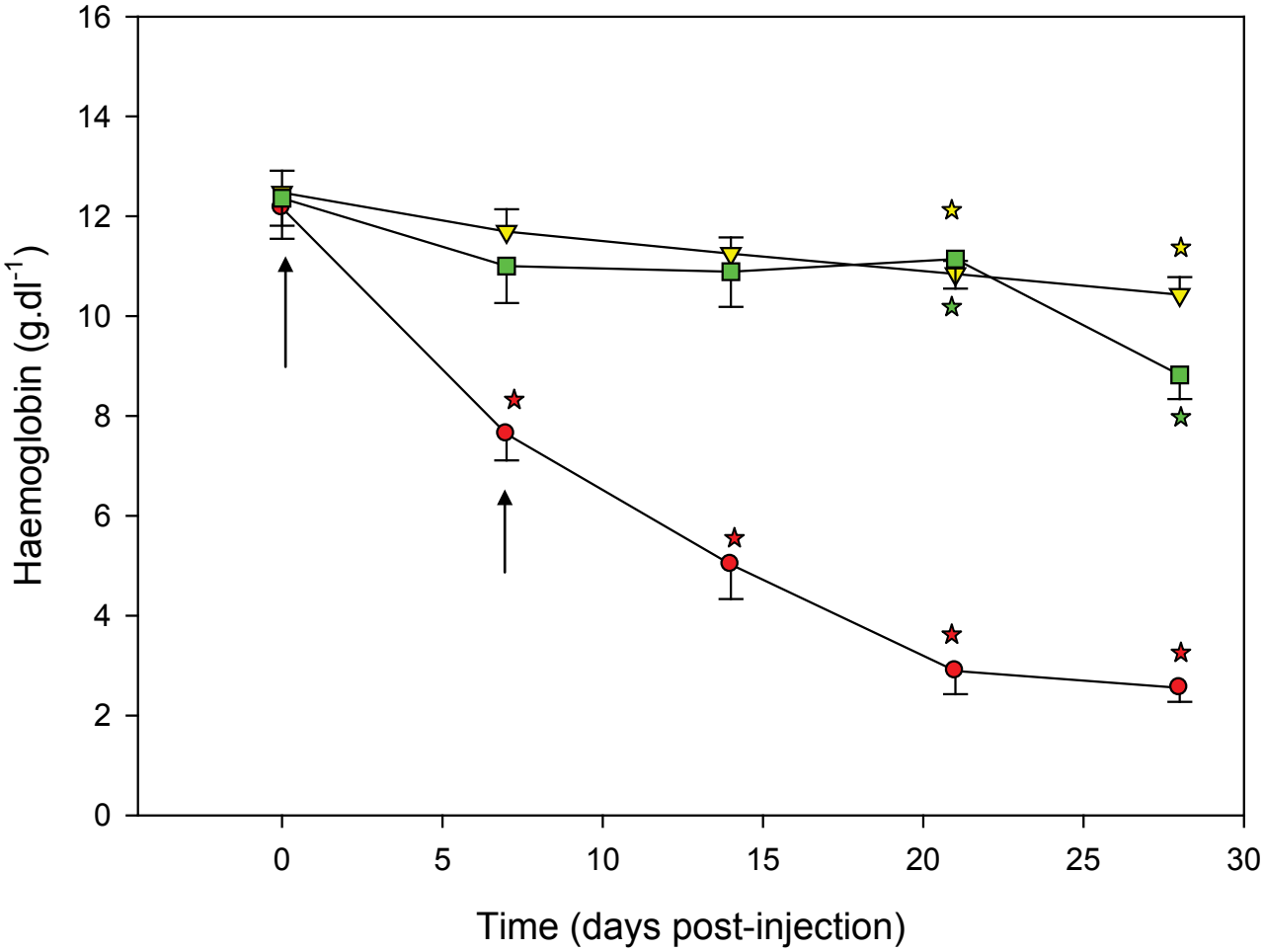

Figure 15- Haemoglobin progression, Atlantic halibut (*Hippoglossus hippoglossus*) experiment. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. Arrows indicate injection dates. Stars indicate values that are significantly different from initial concentrations. In the phenylhydrazine group, all samples had significantly lower haemoglobin than the initial. In both the dimethyl sulfoxide and Cortland's saline-only groups, only the final two samples (days 21 and 28) had significantly different concentrations compared to the initial.

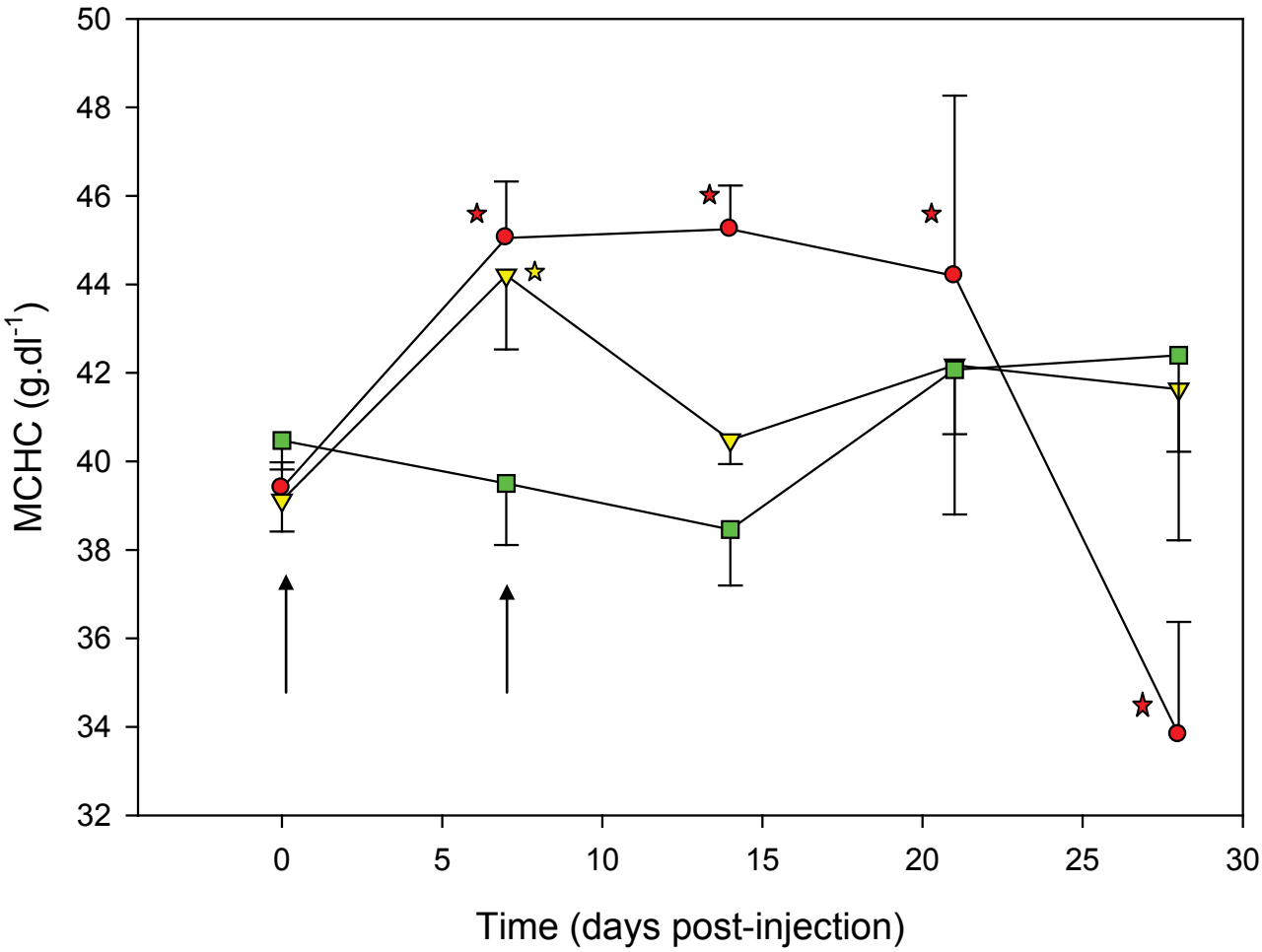

Figure 16- Mean corpuscular haemoglobin concentration changes, Atlantic halibut (*Hippoglossus hippoglossus*) experiment. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. Arrows indicate injection dates, and stars represent values that are significantly different to the initial. All values in the phenylhydrazine group are different from the initial concentration, and there was a significant increase on day 7 in the dimethyl sulfoxide group. There were no differences in the Cortland's saline-only group.

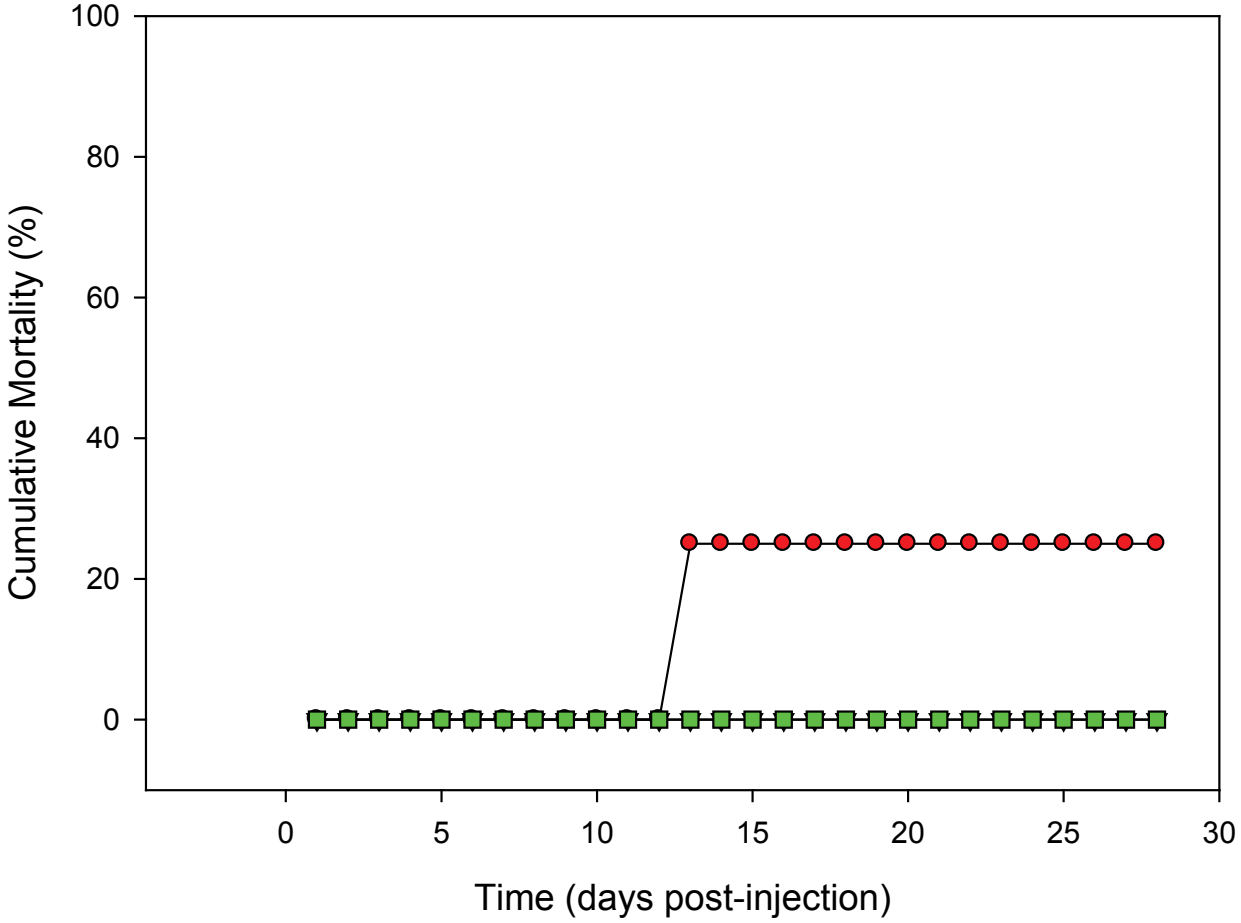

Figure 17- Cumulative mortality, Atlantic halibut (*Hippoglossus hippoglossus*) experiment. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. There were no mortalities in the dimethyl sulfoxide or Cortland's saline-only groups, and a total mortality of 25% in the phenylhydrazine-injected group.

3.1.5 Atlantic salmon

3.1.5.1 Haematocrit

Salmon showed an initial, pre-treatment mean haematocrit packed cell volume of 36.67 ± 1.23- 42.14 ± 1.51%, and final values in the experiment were between 13.10 ± 0.91- 27.67 ± 2.62% (Figure 18). As with the halibut, significant differences in haematocrit percentages only came after the second injection (Figure 18). The phenylhydrazine group saw significant reductions (H_4=84.339, P= < 0.001), with the samples from days 14, 21, and 25 having lower haematocrit than both the initial and day 7 samples. The average haematocrit percentages in the DMSO group were significantly lower (H_4= 43.643, P= < 0.001) than initial values in the samples from days 14, 21, and 25. The same differences were seen in the saline-only group ($F_{4,24}$= 9.571, P= < 0.001). There was a significant difference (H_2 24.935 P= < 0.001) in the terminal haematocrit values between the three treatment groups, with the phenylhydrazine-injected group having a significantly lower haematocrit concentration (Figure 18).

3.1.5.2 Haemoglobin

The initial mean salmon haemoglobin content was between 13.50 $g \cdot dl^{-1}$ ± 0.49 SE- 14.59 ± 0.75, and final mean concentrations for the groups ranged from 2.74 $g \cdot dl^{-1}$ ± 0.16- 8.03 ± 0.90 (Figure 19). Following the second injection, there was a significant (H_4= 77.882, P= < 0.001) reduction in haemoglobin content in the PHZ group (Figure 19). For both the DMSO and saline-only groups, there were significant reductions in haemoglobin in only the fourth and fifth samples (days 21 and 25), when compared to the initial content (DMSO: H_4= 44.318, P= < 0.001; Saline-only: $F_{4,26}$= 11.207, P= < 0.001). Between treatments, the phenylhydrazine-injected group had significantly lower haemoglobin content (H_2= 24.935, P= < 0.001) than both the DMSO and saline-only groups (Figure 19).

Mean Corpuscular Haematocrit Concentration

The MCHC for the phenylhydrazine-injected group varied greatly throughout the experiment (Figure 20). The mean began at a level of 42.17 $g \cdot dl^{-1}$ ± 1.73 SE, and reduced to 36.67 ±0.76 at day 7. Following this, there was a significant (H_4= 52.308, P= < 0.001) increase to 45.71 $g \cdot dl^{-1}$ ± 1.64, a decrease to 30.03 ± 1.45 by day 21, and a further decrease to 25.27 $g \cdot dl^{-1}$ ± 3.48 by day 25. There was less variation in the DMSO group, which started with a concentration of 34.77 $g \cdot dl^{-1}$ ± 0.79, and remained relatively stable until day 21. By day 25, however, there was a significant (H_4= 16.132, P= 0.003) decrease in MCHC, to a low of 28.54 $g \cdot dl^{-1}$ ± 2.63 (Figure 20). There were no apparent significant differences in the saline-only treatment group (H_4= 9.004, P= 0.061). There was a high-point of 41.13 $g \cdot dl^{-1}$ ± 1.09 at day 14, and a low of 32.07 ± 7.29.

3.1.5.3 Mortality

Following injection, there was an overnight loss of 3 fish from the phenylhydrazine group, 6 from the DMSO group, and 1 from the saline-only group (Figure 21). There were no further losses during the experiment, giving a cumulative mortality of 12.5% for the PHZ group, 28.6% for the DMSO group, and 14.3% for the saline-only group.

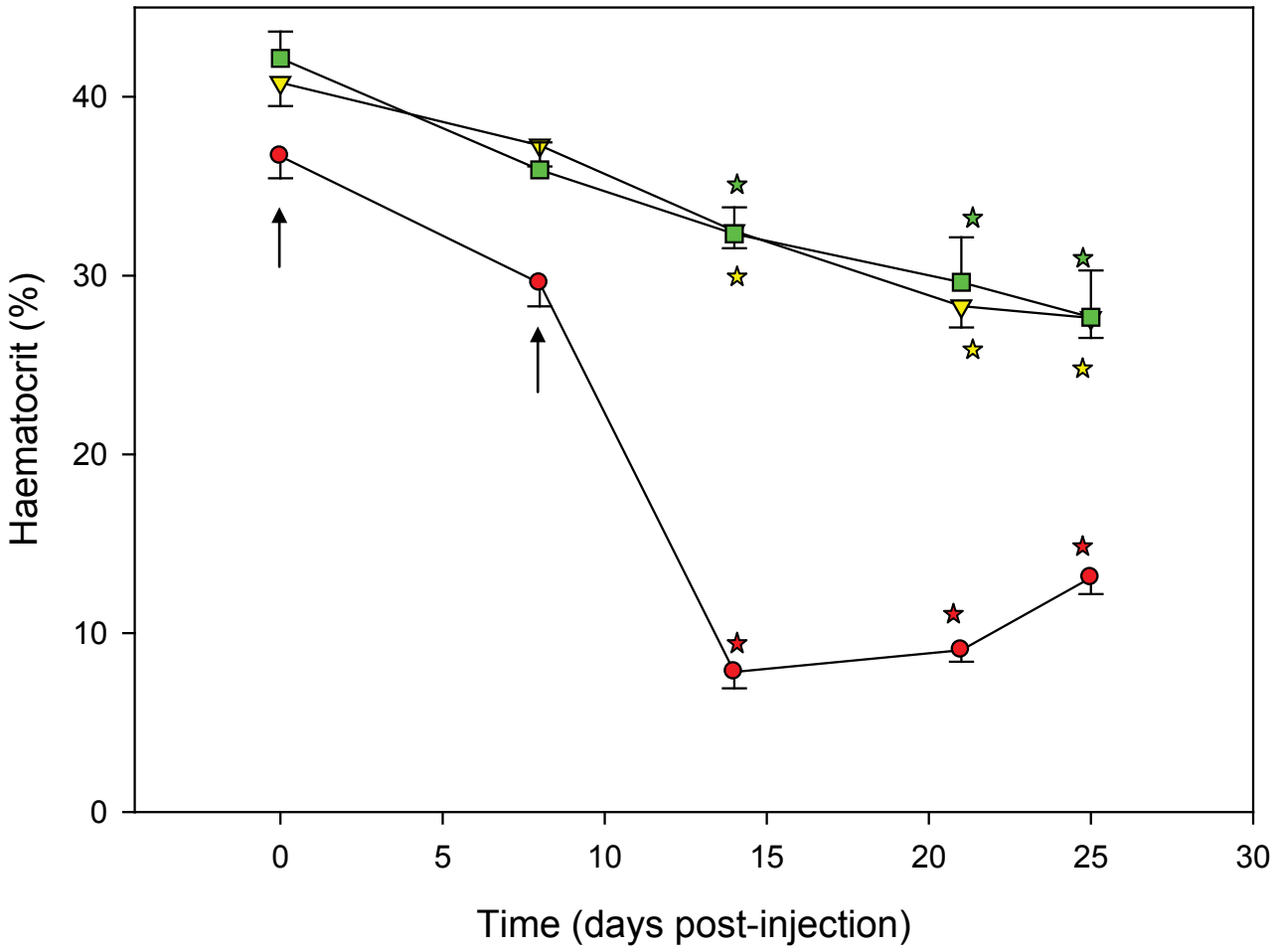

Figure 18- Haematocrit progression- Atlantic salmon (*Salmo salar*) experiment. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline control group by squares. Arrows indicate injection dates. Stars indicate a significant difference from initial concentration. The values at days 14, 21, and 25 are significantly different from initial values in all groups.

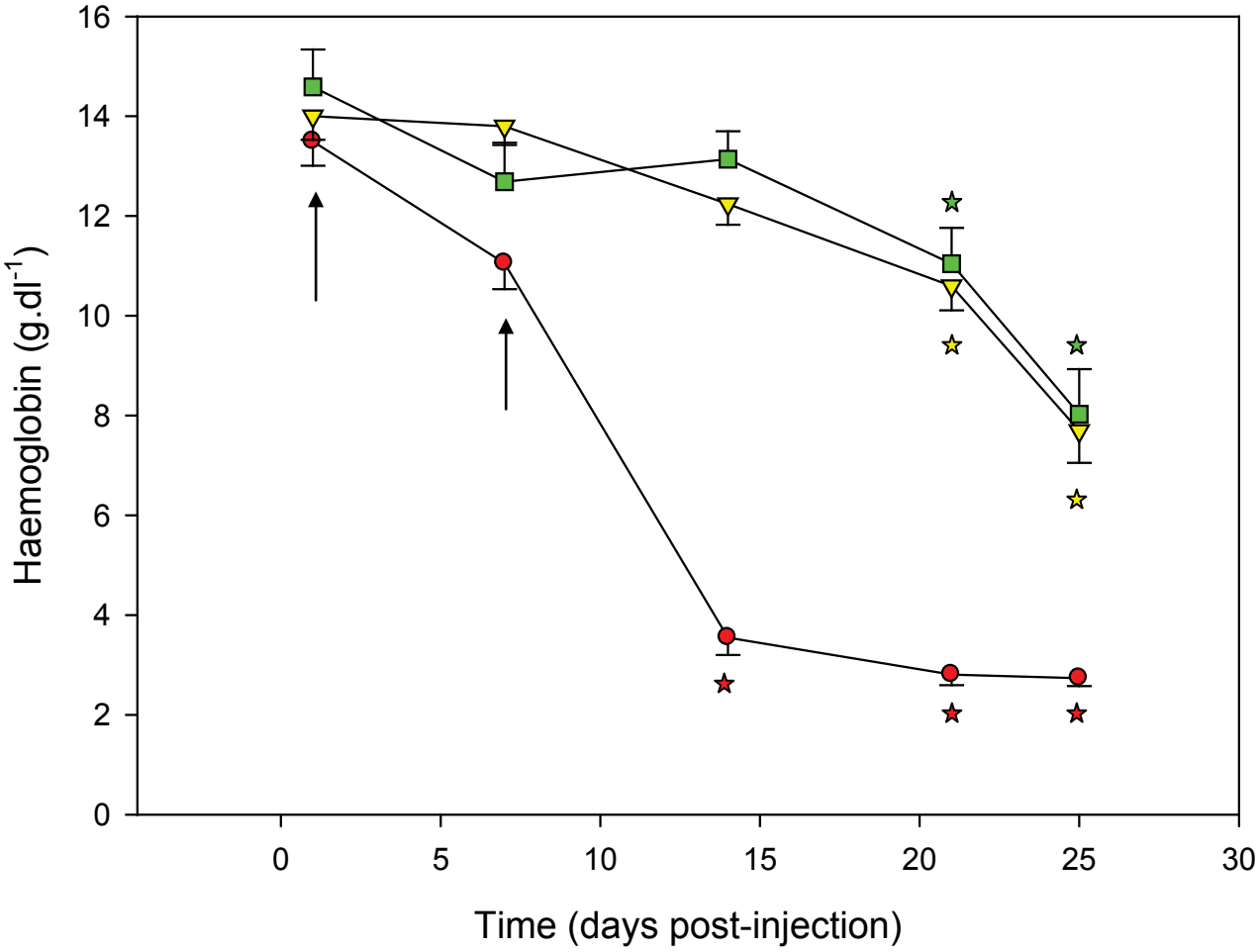

Figure 19- Haemoglobin progression, Atlantic salmon (*Salmo salar*) experiment. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. Arrows indicate injection dates. Stars indicate a significant difference from initial concentration. The haemoglobin content on days 14, 21, and 25 were significantly lower in the phenylhydrazine group. There was a reduction seen in both the dimethyl sulfoxide and Cortland's saline-only groups on days 21 and 25.

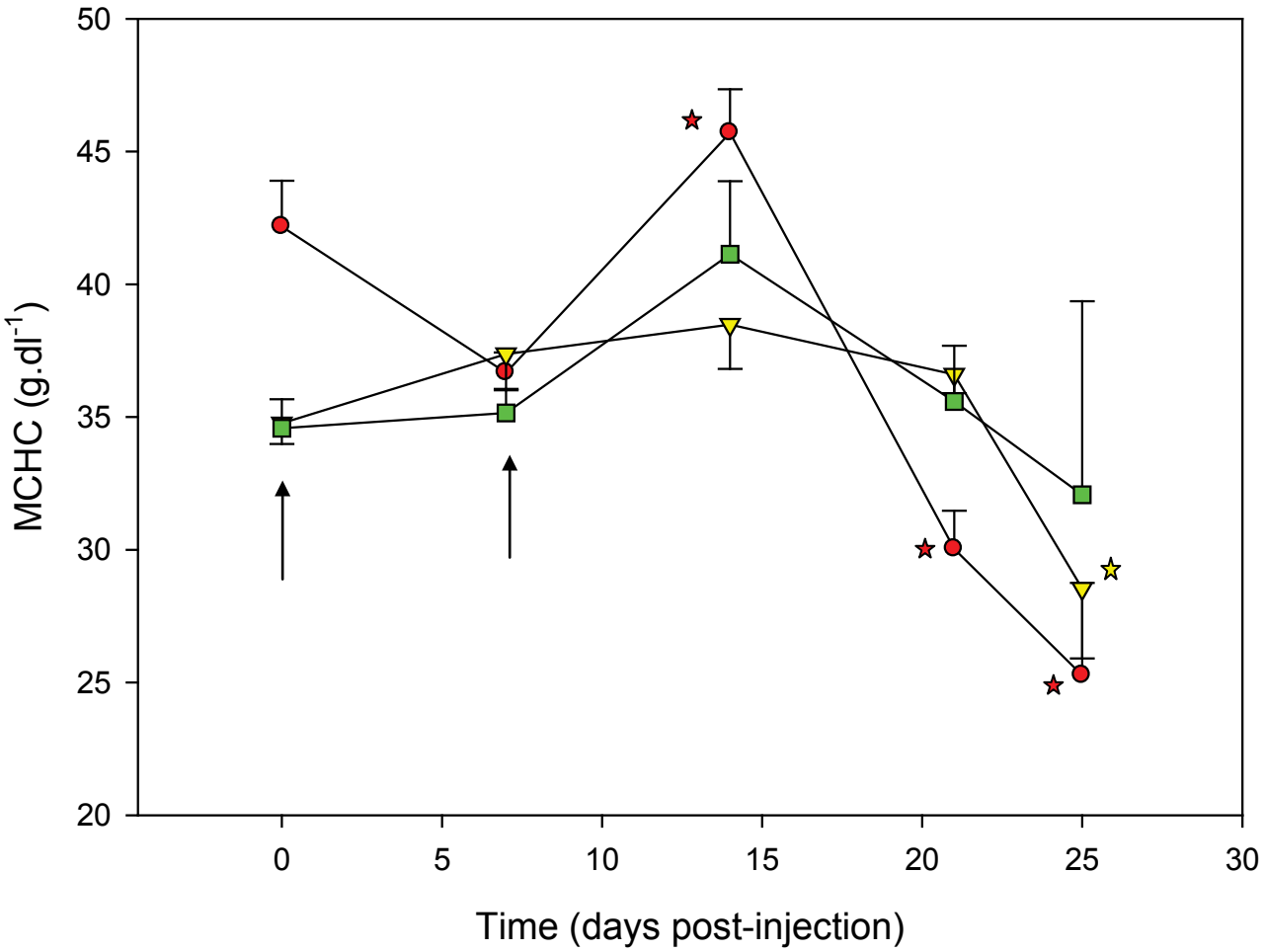

Figure 20- Mean corpuscular haemoglobin concentration values, Atlantic salmon (*Salmo salar*) experiment. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. Arrows indicate injection dates, and stars indicate values that are significantly different. The samples from days 14, 21, and 25 are significantly different from the initial in the phenylhydrazine group. In the dimethyl sulfoxide, there was a reduction at day 25, and there were no differences seen in the Cortland's saline-only group.

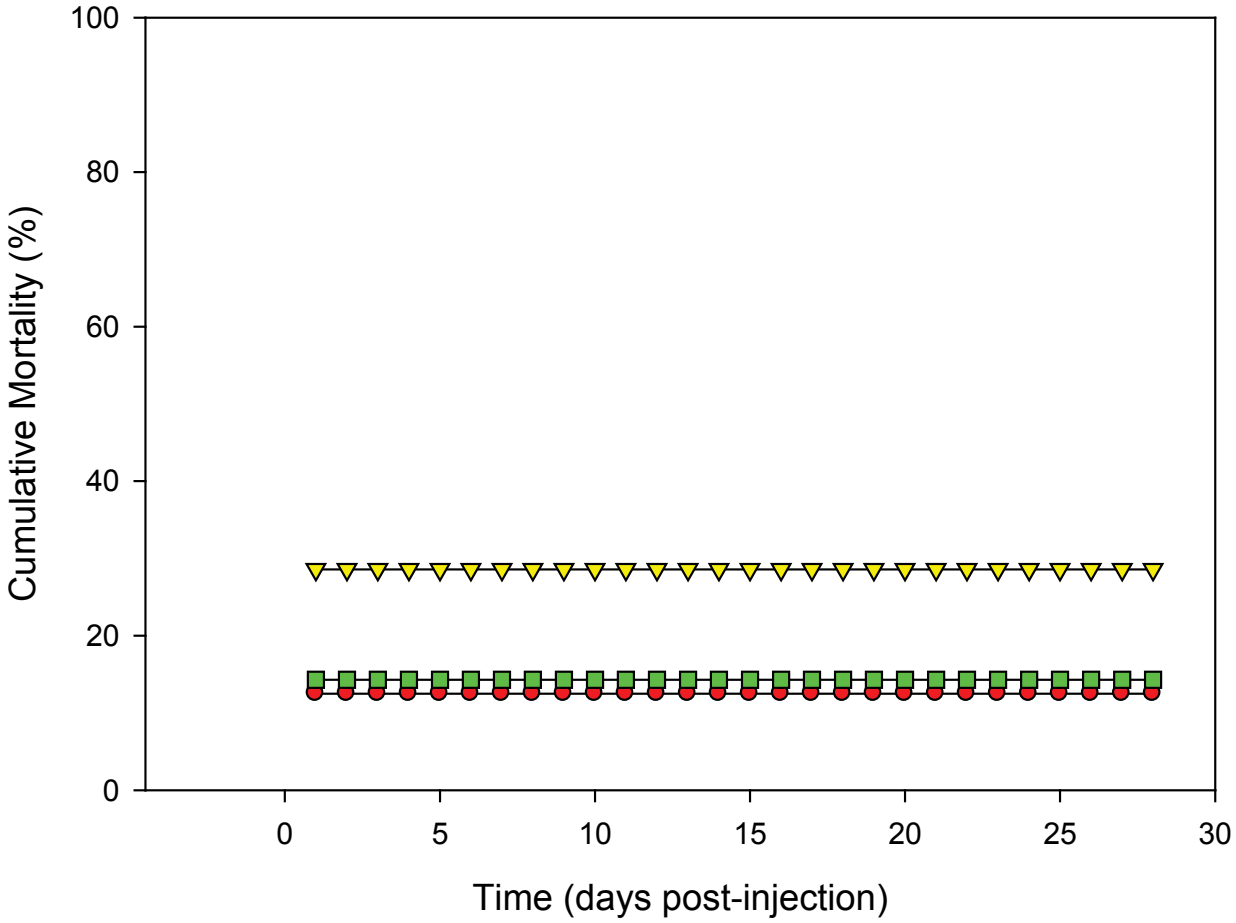

Figure 21- Cumulative mortality, Atlantic salmon (*Salmo salar*) experiment. The phenylhydrazine group is represented by circles, the dimethyl sulfoxide group by inverted triangles, and the Cortland's saline-only group by squares. There was a total mortality of 12.5% in the phenylhydrazine-injected group, 28.6% in the dimethyl sulfoxide group, and 14.3% in the Cortland's saline-only group.

3.2 Lactate and glucose

3.2.1 Atlantic cod

Plasma lactate measurements were made on the initial and terminal samples from Atlantic cod from experiment four (Table 1). In the phenylhydrazine group, there was a significant difference ($U= 83$, $P= 0.030$) between initial and terminal lactate values (3.05 $mmol \cdot l^{-1}$ ± 0.27 SE vs. 2.49 $mmol \cdot l^{-1}$ ± 0.27). There were no differences seen in the DMSO (initial: 5.43 ± 0.89, final: 3.67 $mmol \cdot l^{-1}$ ± 5.56; $U= 138$, $P= 0.064$) or Cortland's saline (initial: 3.71 ± 0.49, final: 2.43 $mmol \cdot l^{-1}$ ± 0.36; $t_8= 1.902$, $P= 0.094$) groups. There were also no significant concentration differences between the final lactate concentrations for any of the treatments ($H_2= 2.857$, $P= 0.240$).

Plasma glucose measurements were also made on the Atlantic cod from experiment four (Table 1). There were no significant differences in the PHZ ($t_{20}= -0.151$, $P= 0.882$), DMSO ($t_{26}= 0.189$, $P= 0.852$) or Cortland's saline ($t_8= 1.180$, $P= 0.272$) groups. The PHZ group started with a concentration of 2.81 ± 0.41 SE and had a final concentration of 2.91 $mmol \cdot l^{-1}$ ± 0.42. The DMSO group progressed from 3.33 ± 0.37 to 3.25 $mmol \cdot l^{-1}$ ± 0.25, while the Cortland's saline group had an initial concentration of 3.45 $mmol \cdot l^{-1}$ ± 0.65 and ended with a final concentration of 2.37 $mmol \cdot l^{-1}$ ± 0.51. Additionally, there were no apparent significant differences between treatments, with regard to the final plasma glucose concentrations ($F_{2,21}= 1.234$, $P= 0.311$).

3.2.2 Atlantic halibut

All of the halibut plasma samples tested had lactate concentrations below the detection threshold of 0.7 $mmol \cdot l^{-1}$ (Table 1). There was a significant reduction ($U= 81$, $P= 0.011$) in

plasma glucose concentration between the initial and final samples in the phenylhydrazine-injected group (Table 1). The average glucose content at the beginning of the experiment was 1.55 mmol·l^{-1} ± 0.27 SE, and had reduced to 0.37 mmol·l^{-1} ± 0.15 by the end. There were also significant reductions in plasma glucose in the DMSO (initial: 1.23 ± 0.14, final: 0.19 mmol·l^{-1} ± 0.10; U= 128.5, P= < 0.001) and Cortland's saline-only groups (initial: 1.62 ± 0.21, final: 0.00 mmol·l^{-1} ± 0.00; U= 0, P= 0.008). In the Cortland's saline only group, all final glucose concentrations were below the detection limit. As there were reductions in each group, there were no significant differences seen between terminal concentrations in each of the treatments (H_2= 3.564, P= 0.168).

3.2.3 Atlantic salmon

The plasma lactate concentrations showed no significant variation between initial and terminal concentrations for neither the phenylhydrazine (initial: 3.82 mmol·l^{-1} ± 0.17, final: 3.76 ± 0.20; U=279, P= 0.564), the DMSO (initial: 4.42 mmol·l^{-1} ± 0.43, final: 3.51 ± 0.27; U= 209, P= 0.102), nor the Cortland's saline only (initial: 5.04 mmol·l^{-1} ± 0.98, final: 3.51 ± 0.24; U= 28, P= 0.366) groups (Table 1). There were, similarly, no significant differences in final lactate concentrations between the treatment groups (H_2= 1.342, P= 0.511).

However, there were significant reductions in plasma glucose in all of the treatment groups. The PHZ-injected group saw a reduction (U= 455, P= < 0.001) from 4.92 mmol·l^{-1} ± 0.17 SE to 3.68 mmol·l^{-1} ± 0.10. The DMSO group had a significant difference (U= 209, P= < 0.001) between the initial concentration of 6.00 mmol·l^{-1} ± 0.23 and the final, 3.27 mmol·l^{-1} ± 0.15. The Cortland's saline-only group also saw a significant reduction (t_{11}= 5.016, P= < 0.001) between the initial (5.80 mmol·l^{-1} ± 0.39) and final (3.12 mmol·l^{-1} ± 0.33) concentrations.

There was no variation (H_2= 4.202, P= 0.122) in the final glucose concentrations when the different treatments were compared to each other.

Table 1- Lactate and glucose concentrations. Data are presented as average ± standard error. The sample size is written subscript after the mean. An asterisk represents a significant difference between initial and final concentrations. ND represents 'no data'. There was a significant difference between initial and final mean lactate concentrations in the phenylhydrazine-injected group of Atlantic cod. The mean plasma glucose concentrations were significantly different in all treatment groups of Atlantic salmon and Atlantic halibut.

Species	**Lactate mmol.l^{-1} (±SE)**		**Glucose mmol.l^{-1} (±SE)**	
Treatment	Initial	Final	Initial	Final
Atlantic Cod				
PHZ	3.05_{15} (±0.27)*	2.49_{7} (±0.27)*	2.81_{15}(±0.41)	2.91_{7} (±0.42)
DMSO	5.43_{15} (±0.89)	3.67_{13} (±0.56)	3.33_{15} (±0.37)	3.25_{13} (±0.25)
Saline	3.71_{6} (±0.49)	2.43_{4} (±0.36)	3.45_{6} (±0.65)	2.37_{4} (±0.51)
Atlantic Salmon				
PHZ	3.82_{24} (±0.22)	3.76_{21} (±0.20)	4.92_{24} (±0.17)*	3.68_{21} (±0.10)*
DMSO	4.42_{21} (±0.43)	3.51_{15} (±0.27)	6.00_{21} (±0.23)*	3.27_{15} (±0.15)*
Saline	5.04_{7} (±0.98)	3.51_{6} (±0.24)	5.80_{7} (±0.39)*	3.12_{6} (±0.33)*
Atlantic Halibut				
PHZ	nd	nd	1.55_{12} (±0.27)*	0.37_{8} (±0.15)*
DMSO	nd	nd	1.23_{12} (±0.14)*	0.19_{11} (±0.10)*
Saline	nd	nd	1.62_{5} (±0.21)*	0.00_{5} (±0.00)*

3.3 Electrolytes

3.3.1 Atlantic cod

The terminal plasma samples from Atlantic cod experiment four were used for these analyses. There were no significant inter-treatment differences ($F_{2,17}$= 1.112, P= 0.354) for plasma sodium (Table 2). The PHZ group had an average concentration of: 178.26 mmol·l^{-1} ± 2.44 SE, DMSO: 174.81 mmol·l^{-1} ± 2.40, and Cortland's saline-only: 181.65 mmol·l^{-1} ± 5.64. In plasma chloride concentrations, the phenylhydrazine-injected group averaged 148.44 mmol·l^{-1} ± 2.60, the dimethyl sulfoxide group 141.81 mmol·l^{-1}± 1.64, and the Cortland's saline-only group 145.91 ± 5.56 mmol·l^{-1}. There was no significant difference between these concentrations ($F_{2,22}$= 2.012, P= 0.160). As for plasma potassium concentrations, there were also no differences apparent between treatments ($F_{2,22}$= 1.669, P= 0.214), with the phenylhydrazine group averaging 6.64 mmol·l^{-1} ± 0.21, the DMSO group 6.03 mmol·l^{-1} ± 0.13, and the Cortland's saline group 6.60 mmol·l^{-1} ± 0.52.

3.3.2 Atlantic halibut

There was a significant difference ($F_{2,22}$= 3.868, P= 0.038) in plasma sodium concentrations between treatment groups, with the phenylhydrazine group having a higher concentration (199.43 mmol·l^{-1} ± 3.70 SE) compared to the DMSO (192.24 mmol·l^{-1} ± 0.88) and Cortland's saline (181.72 mmol·l^{-1} ± 2.08) groups (Table 2). A significant difference ($F_{2,22}$= 14.286, P= < 0.001) between the three treatment groups with regard to plasma chloride was observed. The phenylhydrazine group had a mean content of 171.19 mmol·l^{-1} ± 3.92, the mean of the dimethyl sulfoxide group was 165.82 mmol·l^{-1} ± 3.66, and the Cortland's saline group had a mean of 136.2 mmol·l^{-1} ± 2.98. The difference in these parameters, however, is between the

Cortland's saline control group and the others. When this group was omitted, there was no significant difference between the PHZ and DMSO treatments with regard to sodium (t_{17}= 1.380, P= 0.816) and chloride (t_{17}= 0.985, P= 0.338). There was no significant difference (H_2= 5.165, P= 0.076) between groups when plasma potassium was analyzed (Table 2). The PHZ group had a mean of 7.33 mmol·l^{-1} ± 0.28, the DMSO group contained 8.21 mmol·l^{-1} ± 0.37, and the Cortland's saline group averaged 6.71 mmol·l^{-1} ± 0.46.

3.3.3 Atlantic salmon

There was no significant difference ($F_{2,40}$= 0.630, P= 0.538) in plasma sodium between groups (Table 2). The PHZ group had a concentration of 168.00 mmol·l^{-1} ± 0.87 SE, the DMSO group 167.60 mmol·l^{-1} ± 0.88, and the Cortland's saline group a concentration of 166.00 mmol·l^{-1} ± 1.61. There were also no significant differences in plasma chloride ($F_{2,40}$= 1.823, P= 0.175). In this parameter, the phenylhydrazine group had a mean concentration of 136.11 mmol·l^{-1} ± 0.92, while the DMSO group contained on average 135.32 mmol·l^{-1} ± 0.87, and the Cortland's saline group had 132.75 mmol·l^{-1} ± 1.44. Similarly, no differences were seen in plasma potassium ($F_{2,40}$= 2.305, P= 0.114) between the treatment groups, where the PHZ group contained 6.64 mmol·l^{-1} ± 0.21, the DMSO group had 6.03 mmol·l^{-1} ± 0.13, and the Cortland's saline group averaged 6.60 mmol·l^{-1} ± 0.52 plasma potassium.

Table 2- Terminal plasma electrolyte concentrations. Values are presented as mean ± standard error. Sample size is noted in subscript on the treatment type in column 1. An asterisk represents a significant difference. Mean plasma sodium and chloride levels in the Atlantic halibut Cortland's saline-only group were significantly lower than both the phenylhydrazine and dimethyl sulfoxide groups.

Species	Sodium $mmol.l^{-1}$ (±SE)	Chloride $mmol.l^{-1}$ (±SE)	Potassium $mmol.l^{-1}$ (±SE)
Atlantic Cod			
PHZ_7	178.26 (± 2.44)	148.44 (± 2.60)	4.59 (± 0.16)
$DMSO_{12}$	174.81 (± 2.40)	141.81 (± 1.64)	4.07 (± 0.19)
Saline4	181.65 (± 5.64)	145.91 (± 5.56)	4.32 (± 0.33)
Atlantic Salmon			
PHZ_{20}	168.00 (± 0.87)	136.11 (± 0.92)	6.64 (± 0.21)
$DMSO_{15}$	167.60 (± 0.88)	135.32 (± 0.87)	6.03 (± 0.13)
$Saline_6$	166.00 (± 1.61)	132.75 (± 1.44)	6.60 (± 0.52)
Atlantic Halibut			
PHZ_8	199.43 (± 3.70)	171.19 (± 3.92)	7.33 (± 0.28)
$DMSO_{11}$	192.24 (± 3.52)	165.82 (± 3.66)	8.21 (± 0.37)
$Saline_4$	181.72 (± 2.08)*	136.20 (± 2.98)*	6.71 (± 0.46)

3.4 Differential blood counts

The relative proportions of lymphocytes, thrombocytes, monocytes, and neutrophils were measured for each of the sample groups per species (Tables 3, 4). The before- and after-treatment proportions within each treatment group were compared using z-tests, but there were no significant differences. The between-treatment proportions (for end-point samples) were also tested, but had similarly non-significant results. There was, however, a marked increase in the proportion of immature erythrocytes in each of the phenylhydrazine-treated groups post-injection.

Table 3- Differential blood smear data, initial proportions. Values for white blood cells are presented as percentages of total number ± SE. Immature erythrocyte data is presented as percentage of total erythrocytes present. Subscripts on the treatments in column 1 denote the sample size. There were no significant changes in white blood cell proportions throughout the experiments. There was an increase in the relative proportion of immature erythrocytes in the phenylhydrazine-treated groups of all species.

Species **Treatment**	**Lymphocytes %**	**Thrombocytes %**	**Monocytes %**	**Neutrophils %**	**Immature Erythrocytes %**
Atlantic Cod	Before (±SE)	Before (±SE)	Before (±SE)	Before (±SE)	Before
PHZ_7	58.82 (7.40)	26.11 (5.09)	14.95 (2.76)	0.13 (0.13)	< 5
$DMSO_9$	45.96 (1.64)	28.37 (2.92)	25.47 (2.38)	0.01 (0.01)	0
$Saline_2$	58.97 (3.53)	20.93 (3.11)	20.10 (6.64)	0.00 (0.00)	0
Atlantic Salmon					
PHZ_9	53.67 (4.09)	29.53 (5.05)	11.12 (2.15)	5.68 (1.92)	< 5
$DMSO_7$	53.11 (4.34)	32.50 (5.03)	9.05 (2.10)	5.33 (1.17)	< 5
$Saline_6$	56.34 (2.71)	29.83 (4.40)	7.10 (2.74)	6.73 (1.83)	0
Atlantic Halibut					
PHZ_7	72.26 (4.31)	22.51 (4.18)	5.08 (1.34)	0.16 (0.16)	0
$DMSO_7$	72.93 (4.01)	21.63 (3.29)	5.39 (1.34)	0.01 (0.01)	0
$Saline_3$	62.13 (10.20)	29.20 (4.81)	8.68 (5.47)	0.00 (0.00)	0

Table 4- Differential blood smear data, final proportions. Values for white blood cells are presented as percentages of total number ± SE. Immature erythrocyte data is presented as percentage of total erythrocytes present. Subscripts on the treatments in column 1 denote the sample size. There were no significant changes in white blood cell proportions throughout the experiments. There was an increase in the relative proportion of immature erythrocytes in the phenylhydrazine-treated groups of all species.

Species **Treatment**	**Lymphocytes %**	**Thrombocytes %**	**Monocytes %**	**Neutrophils %**	**Immature Erythrocytes %**
Atlantic Cod	After (±SE)	After (±SE)	After (±SE)	After (±SE)	After
PHZ_7	38.31 (6.95)	21.52 (5.97)	39.92 (4.13)	0.25 (0.16)	95
$DMSO_9$	53.52 (2.93)	19.86 (1.79)	26.62 (2.85)	0.00 (0.00)	5
$Saline_2$	53.15 (9.86)	23.16 (6.72)	23.69 (3.14)	0.00 (0.00)	< 5
Atlantic Salmon					
PHZ_9	48.28 (4.42)	16.42 (4.46)	22.24 (4.77)	13.06 (3.52)	85
$DMSO_7$	64.90(5.09)	19.03 (3.39)	9.21 (1.64)	6.86 (1.66)	< 5
$Saline_6$	61.41 (6.44)	24.99 (7.82)	8.17 (2.78)	5.43 (1.84)	< 5
Atlantic Halibut					
PHZ_7	83.07 (2.79)	7.11 (0.98)	9.75 (3.24)	0.07 (0.07)	90
$DMSO_7$	79.51 (8.03)	7.56 (1.81)	12.63 (6.90)	0.30 (0.20)	< 5
$Saline_3$	75.59 (9.44)	7.24 (4.39)	17.17 (10.52)	0.00 (0.00)	< 5

4.0 Discussion

4.1 Reduction of haematocrit and haemoglobin, mortality

4.1.1 Atlantic cod experiments one and two

The purpose of the first two experiments was to determine an appropriate dose of phenylhydrazine to induce a stable anaemia in Atlantic cod, while minimizing the subsequent mortality. In experiment one, one dose of 10 $\mu g \cdot g^{-1}$ proved too concentrated, as all of the fish in the test group died. For experiment two, several doses of the drug were used to determine a more suitable concentration that induced anaemia more gradually, and reduced the cumulative mortality. The 8 $\mu g \cdot g^{-1}$ group also had 100% mortality, so the other options were 2.5 or 5 $\mu g \cdot g^{-1}$. There was an unacceptably high mortality in the 5 $\mu g \cdot g^{-1}$ group, while the haematocrit was not sufficiently reduced in the 2.5 $\mu g \cdot g^{-1}$ group. The initial blood samples from fish that died throughout the experiment were included in subsequent analyses, as they were still representative of baseline values. These mortalities immediately following injection were the reason that there was an initial mortality rate of 22%, 25%, and 28.6% in the 0 $\mu g \cdot g^{-1}$, 5 $\mu g \cdot g^{-1}$, and 10 $\mu g \cdot g^{-1}$, respectively, which inflated the mortality figures.

Considering the degree of haematocrit reduction as well as mortality numbers, a dose of 3 $\mu g \cdot g^{-1}$ was optimal, and was therefore used for the subsequent experiments, inducing a sustained anaemia over the course of two weeks. This dose is much lower than the concentrations used previously for other species, particularly salmonids (rainbow trout: 10 $\mu g \cdot g^{-1}$- Simonott and Farrell, 2007, McClelland, Dalziel, Fragoso, and Moyes, 2005; chinook salmon12.5 $\mu g \cdot g^{-1}$- Smith et al., 1971). However, Cameron and Wohlschlag (1969) used the same concentration for pinfish, with success in reducing both haematocrit and

haemoglobin, though the average concentrations for these, 18.8% and 3.70 $g \cdot dl^{-1}$, respectively, were higher than those achieved for Atlantic cod in the present study.

4.1.2 Atlantic cod experiments three and four

In Atlantic cod experiment 3, there was a decrease in both haemoglobin and haematocrit between days zero and 7, but an increase in MCHC. This suggested that there may have been more haemoglobin per erythrocyte, and the fish were essentially making their blood more efficient. This would be beneficial to the fish, as it would lessen the incidence of hypoxia. However, in a study on rats, Criswell, Sulkanen, Hochbaum, and Bleavins (2000) found that with phenylhydrazine-induced anaemia, there was an increase in free haemoglobin in the plasma, which increased the MCHC. This potentially explained the increase in this study. However, by the next sample instance, the MCHC had significantly reduced. This can be explained by an increase in immature erythrocytes, which have less haemoglobin per cell than do mature red blood cells (Valenzuela, Silva, and Klempau, 2006). The mean corpuscular haemoglobin concentration did not appear to follow a discernible pattern in relation to the haematocrit and haemoglobin content and is not a reliable gauge for interpreting the severity of anaemia. As with experiments one and two, the mortality data for experiments three and four was artificially inflated due to initial, post-injection losses.

In experiment four, the PHZ-group experienced haematocrit reduction by day 14 similar to that which was experienced in experiment 3 (11.85% ± 3.21 in experiment 4; 8% ± 0.00 in experiment 3), then started to show improvement by day 21, a trend that was mirrored by the haemoglobin concentration. The mean corpuscular haemoglobin concentration showed no significant changes in experiment four. The terminal values for haematocrit and haemoglobin in the phenylhydrazine-injected group was still significantly reduced three

weeks post-injection. Smith et al. (1971) undertook a much longer-term investigation of the red blood cell counts, haematocrit, and haemoglobin of phenylhydrazine-injected Chinook salmon (*Onchorynchus tshawytscha*). The fish were intraperitoneally injected with 12.5 $\mu g \cdot g^{-1}$ PHZ and blood samples were taken at 4, 8, 10, 15, 28, 39, and 95 days post-injection (Smith et al., 1971). The blood parameters of injected fish only began to near those of the controls at 95 days post-injection, although haemoglobin content was still lower (5.90 vs. 6.70 $g \cdot dl^{-1}$ control).

4.1.3 Atlantic halibut

In the Atlantic halibut experiment, there was a dramatic decrease in both haematocrit and haemoglobin in the phenylhydrazine-injected group, with a corresponding significant increase in MCHC. The MCHC increased by day 7, and remained increased through day 21, after which it dropped significantly by day 28, which corresponds with the levelling of haematocrit and haemoglobin concentrations. As with the cod experiments, the elevated MCHC in this experiment could either indicate an actual increase in the amount of haemoglobin per erythrocyte or a false high influenced by free haemoglobin in the plasma (as seen in Criswell et al., 2000), although this was not investigated in the present study. This was the only apparent pattern for MCHC in the experiment, as there were no differences in the Cortland's saline-only group, and only a transient post-injection increase in the DMSO group. The low mortality seen in this experiment (3 fish of the PHZ group, for a total of 25%) indicated that Atlantic halibut are able to deal with an anaemic condition. Wood et al. (1979) demonstrated that the starry flounder (*Platichthys stellatus*) was tolerant of experimental anaemia, surviving with haematocrit levels as low as 1%.

4.1.4 Atlantic salmon

The haematocrit and haemoglobin profiles for the salmon experiment roughly followed that of the halibut- for the PHZ group, a slight decrease after the first injection, and further decrease after the second dose, followed by a slight increase in haematocrit, while the haemoglobin concentration stabilized. There was no apparent effect of treatment on the DMSO or Cortland's Saline-only groups and the depression of haemoglobin and haematocrit levels at the end of the experiment were likely due to repetitive blood sampling. There was a substantial increase in MCHC after the second injection (day 14), which then drastically reduced for the samples at days 21 and 25. The increase in haematocrit with concurrent decrease in haemoglobin suggests that the red blood cells were predominately immature, and contained less haemoglobin. Smith et al. (1971) saw a similar lag in haemoglobin recovery in phenylhydrazine-injected Chinook salmon for upwards of 95 days, which suggested that the haemoglobin content of the blood does not return to control levels until the erythrocytes have matured. Again, as with the Atlantic cod experiments, mortality numbers for this experiment were inflated due to the inclusion of baseline data from fish that died on injection day.

4.2 Effects on lactate and glucose

4.2.1 Plasma lactate

It was expected that there would be an increase in plasma lactate concentration with the progression of anaemia. This phenomenon has been well documented as occurring in many experiments involving anaemia (Wood et al., 1982; Mansell, Powell, Ernst, and Nowak, 2005; Olsen et al., 1992). In the study by Wood et al. (1982), rainbow trout and starry flounder were made progressively anaemic via blood removal- through either dorsal/ventral

aortic cannulae in the case of trout, or caudal vein cannulae in the case of flounder- over a period of 4- 6 days in order to study the effects on acid-base regulation. The experimenters noted an overall trend of increased lactate with decreased haematocrit for the rainbow trout, although it was quite variable between individual fish (Wood et al., 1982). However, there was no such trend seen in the flounder, which was also the case with the same species and similar experimental design investigated by Wood et al. previously (1979). These data are in agreement with the Atlantic halibut data of the current study, where all fish had lactate levels that were below detection, and there was no apparent increase.

Increases in lactate concentration with an anaemic state have been documented in studies of which the anaemia itself was not the main focus. Mansell et al. (2005) undertook an investigation of the effects on kingfish, *Seriola lalandi* Valenciennes, of the monogenean *Zeuxapta seriolae*. This is a gill fluke that feeds directly on the blood of the infected fish. In the experiment, there was a significant decrease in haemoglobin correlated with an increase in parasite load. There was also a marked increase in lactate, which inversely correlated with the haemoglobin levels (Mansell et al., 2005). Olsen et al. (1992) looked into the changes of lactate in Atlantic salmon infected with the ISA, infectious salmon anaemia virus. Much like Wood et al. (1982), Olsen et al. (1992) observed a high variance between individual fish with regard to blood lactate concentrations in Atlantic salmon, although an overall increase in lactate was noted with a decrease in haemoglobin.

In all of the species investigated in the current study, there was a trend toward lower plasma lactate concentration at the end of the experiment as compared to initial concentrations. This is despite the significant reductions of haematocrit and haemoglobin in the drug-injected

groups. The only significant lactate reduction, however, was in the phenylhydrazine-injected group of Atlantic cod (Table 1). This group had a mean initial lactate content of 3.05 $mmol \cdot l^{-1}$ ± 0.27 SE, and a final content of 2.49 $mmol \cdot l^{-1}$ ± 0.27. Although the reduction was significant, it was small. It is worthy of noting, as well, that there were 15 samples in the initial group, and only 7 samples in the final group. It is plausible that the fish that died throughout the experiment were those that had elevated plasma lactate levels. Additionally, there was a noticeable lethargy of the phenylhydrazine-injected fish, so there may have been some behavioural modifications in order to reduce energy demand.

4.2.2 Plasma glucose

There were no significant changes in plasma glucose between initial and final samples for Atlantic cod in any treatment group, yet there were decreases in all treatment groups in both the Atlantic halibut and Atlantic salmon experiments (Table 1). The fact that there were no inter-treatment differences, however, indicates that the reduction of plasma glucose was not a result of treatment. The hypoglycaemia could be a consequence of reduced in food intake during the experiment.

4.3 Changes in electrolytes

There were no significant differences in final (end of experiment) plasma electrolyte concentrations for any of the treatment groups for salmon or cod. This indicated that the anaemia did not appear to have a major osmoregulatory effect in these species. Similar results have been reported under hypoxic conditions in other species. Avilez, Altran, Aguiar, and Morales (2004) exposed *Brycon cephalus* Matrinxa to environmental nitrite (at concentrations of 0.2, 0.4, 0.6 $mg \cdot l^{-1}$), which has been known to have haemolytic effects.

Additionally, the nitrite causes the formation of methaemoglobin, which is a non-functioning form of haemoglobin for oxygen transport. There were, however, no major disruptions in ion balances in the experiment (Avilez et al., 2004).

In an experiment by Wood and Randall (1971), southern flounder (*Paralichthys lethostigma*) were made anaemic via serial bleeding, and the effects of anaemia on ion exchange were studied. They found that even though the branchial sodium efflux rate was reduced, there was no change in plasma sodium levels (Wood and Randall, 1971), suggesting that blood electrolyte levels are defended. In the current Atlantic halibut experiment, there was a marked effect of treatment on mean plasma sodium and chloride concentrations. The Cortland's saline-only group had significantly lower concentrations of these ions when compared to the phenylhydrazine and dimethyl sulfoxide groups. There was, however, no difference between the PHZ and DMSO groups. As the common element between these groups was the dimethyl sulfoxide, it can be inferred that it may be this compound that caused the difference, and the electrolyte differences were not related to the anaemia. Indeed, while dimethyl sulfoxide is a common biological solvent owing to its amphipathic nature, it is not without its side-effects (Santos, Figueira-Coelho, Martins-Silva, and Saldanha, 2003). There is a potential osmotic effect of DMSO (Ellison, Velazquez, and Wright, 1984; Santos, Figueira-Coelho, Saldanha, and Martins-Silva, 2002), which can lead to an elevation of plasma electrolyte concentrations, particularly sodium and potassium (Santos et al., 2002). The Atlantic halibut may have been sensitive to the effects of dimethyl sulfoxide.

4.4 Differential blood counts

A differential blood count is a useful tool to investigate the potential immune system effects of a treatment. In the current study, differential blood counts were performed to characterize the effects on white blood cell proportions during phenylhydrazine-induced anaemia. A study by Dornfest et al. (1990) investigated the mitogenic effects of phenylhydrazine on rat lymphocytes. The test animals were injected with a single dose of phenylhydrazine, which induced haemolytic anaemia. The anaemia was noted as most severe after 2- 4 days. Leukocytosis was apparent, and at its height 4-6 days post-injection, with lymphocytes and monocytes accounting for the bulk of the white blood cells. All of the blood parameters investigated returned to baseline levels after 11 days. They concluded that phenylhydrazine caused an increase in lymphoid cells, and was therefore capable of being an immunostimulant for the rat (Dornfest et al., 1990).

In the current study, initial (pre-injection) and terminal blood smears were used for differential blood count characterization. The terminal samples were obtained after 21 days for Atlantic cod, 28 days for Atlantic halibut, and 25 days for Atlantic salmon. There was no evidence of a significant difference between initial and final white blood cell proportions. There are many possible explanations for this observation. The small sample numbers used in this study- between 2 and 9 individuals per group- could surely have an effect on the robustness of the data. Additionally, it is possible that the height of leukocytosis had passed by the time the final blood samples had been taken. Dornfest et al. (1990) reported that blood parameters in the rat returned to normal 11 days post-injection. While the haematocrit and haemoglobin values in the current study were still decreased at the end of the experiment, they were showing a trend towards increasing. It is possible that the leukocyte counts were

altered earlier in the experiment, but had returned to close to initial levels by the final sampling date (14-21 days after final injections).

Furthermore, Dornfest et al. (1986) noted differences in peripheral blood count results when an electronic vs. manual method was employed. In that study, blood samples from phenylhydrazine-injected rats was investigated using both electric cell counters, and manual means- hemocytometers with light microscopy. There was an apparent leukocytosis when the electric cell counter was used, but it was not detected by manual counting (Dornfest et al., 1986). When the cells were investigated under 400X light microscopy, more leukocytes were visible in the field.

The haematological effects of anaemia can vary greatly between cases, depending on the cause. Between different pathogens, even, there can be varying pathologies. While the common element is a reduction in haematocrit and packed cell volume, infectious salmon anaemia, for example, can also cause a severe leucopenia (Thorud and Djupvik, 1988). There is also evidence that infectious hematopoietic necrosis can also cause leucopenia (Amend and Smith, 1975). This disease presents (in rainbow trout) with a reduction in neutrophils, but an increase in lymphocytes, and monocytes are not affected. There is also evidence of acid-base and ion disruptions, with blood chloride and osmolality being reduced.

The effects of a haemorrhagic disease, however, are different. There are still instances of electrolyte imbalances, but the changes are different. Rehulka (2003) states that viral haemorrhagic septicaemia (VHS) can have haematological effects such as decreased total protein and creatinine as well as lower levels of blood glucose and sodium. There can also be increases in blood potassium and lactate dehydrogenase levels (Rehulka, 2003). Generally,

infections with parasites and lice, and certain viruses like VHS cause a lower volume of blood in circulation, which can have effects on ion, acid-base, and water balance (Roberts and Rodger, 2001). Experimentally-induced anaemia, therefore, is a useful tool for studying the physiological effects associated with anaemia-causing diseases, whether they are haemolytic or haemorrhagic in nature.

5.0 Conclusions

Phenylhydrazine can be used to induce a stable and reproducible anaemia in each of the species studied. Atlantic cod reach anaemic state after one intraperitoneal injection of 3 $\mu g \cdot g^{-1}$ phenylhydrazine, while two injections, one week apart, work best for Atlantic halibut and salmon. The significant reductions in plasma lactate and glucose seen in the experiments were not between-treatment, so phenylhydrazine did not appreciably affect these parameters. Future experiments to this extent should include analysis of blood gases to determine if a hypoxia is incurred. Additionally, anaemia does not appear to have a significant effect on plasma electrolytes in Atlantic cod or salmon. There is a potential effect on plasma sodium and chloride concentrations in Atlantic halibut. The potential effects of dimethyl sulfoxide on these parameters, however, cannot be discounted. There was no apparent effect of phenylhydrazine-induced experimental anaemia on the relative leukocyte proportions of Atlantic cod, Atlantic halibut, or Atlantic salmon.

6.0 References

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